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## Clinical and Experimental

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# A NOTE ON TRYPTOPHANE AND PELLAGROUS GLOSSITIS*

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IOWA CITY, IOWA

## INTRODUCTION

FOUR years elapsed between the discovery that tryptophane might be a precursor of nicotinic acid in the nutrition of animals[1] and the demonstration that it had some therapeutic virtue in human pellagra.[2] This is an extreme contrast to the whirlwind of clinical reports of the effects of nicotinic acid on pellagra which followed the discovery by Elvehjem and co-workers that nicotinic acid prevented or relieved canine black tongue.[3] Meanwhile, Sarett and Goldsmith[4] demonstrated that tryptophane and nicotinic acid had an essential relationship in persons malnourished but without diagnostic evidence of pellagra. Because the incidence of pellagra has declined so much in recent years[5] very few subjects with acute pellagra have been available for clinical testing. In the only report of the use of tryptophane in human pellagra the subjects had been on a grossly inadequate diet for many months and the control diet contained only 4.2 mg. of nicotinic acid and 0.07 Gm. of tryptophane. Improvement was rather slow when tryptophane was given in 2 Gm. doses three times daily. Glossitis improved slightly after twenty-four hours and did not clear until seventy-two hours. Pellagra, while distinctly uncommon, has been reported in Iowa.[6] During the last thirty months we have had under observation only two patients with typical pellagrous glossitis and they had no dermatitis or other diagnostic stigma. Their over-all nutritional state was much better than the nutrition of those reported previously.[2] To gain more information on quantitative aspects of the tryptophane-nicotinic acid requirement in man, we used a diet less deficient in nicotinic acid, gave smaller doses of tryptophane, and obtained clear-cut results very rapidly. This paper records the observations in the two subjects.

## MATERIALS AND METHODS

*Case Reports.—*

CASE 1. E. K., a 57-year-old white man, was admitted to the Medical Service of the University Hospitals on Feb. 19, 1949, and was discharged on April 26, 1949 (Table I). His only serious illness had been typhoid fever at the age of 11 years. He had a long history of inadequate diet and had been addicted to alcohol for many years. He had felt well, however, and had been able to work as a bricklayer until eight months before admission, when he noticed increasing fatigue, progressive weakness of the legs, and numbness and tingling of the feet. His feet swelled toward evening and they dragged and were difficult to control. He had some shortness of breath and a moderately productive cough

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for about five weeks. He had been unable to work or to go out to eat for several weeks and although relatives brought him food he could eat only a few bites at a time. He had lost 25 pounds in six months.

On examination he was well developed and fairly well nourished. There was advanced cheilosis, and the tongue was swollen, very smooth, and red. There were several punctate hemorrhages in the fundus of the right eye. There were no vascular spiders or palmar erythema. His heart was not enlarged, but there was a soft systolic murmur over the aortic area. The pulse rate was 70 and the blood pressure was 160/95. The abdomen was soft and no masses were felt. There was a decrease in anal sphincter tone, and moderate atrophy and loss of strength in all leg muscles. He had bilateral footdrop. The skin of the legs and feet was atrophic. Squeezing the calf muscles and feet was painful. The peripheral vessels were moderately sclerotic.

*Laboratory Data:* The red cell count was 4.87 million and leucocyte count 4,650 per cubic millimeter. The differential count was normal. The hemoglobin was 13.6 Gm. per 100 milliliters. The urine on one occasion had a trace of sugar but later specimens were negative, and both oral and intravenous glucose tolerance tests were normal. Blood Kline and Kolmer reactions were negative.

TABLE I. CASE 1, E. K.

| | DATE | DIET | GLOSSITIS | URINE VOLUME | URINE CREATININE | URINE THIAMINE | URINE NIACIN |
|---|---|---|---|---|---|---|---|
| | 19 Feb. | General | +++ | | | | |
| | 20 | Control | +++ | | | | |
| | 21 | Control | +++ | | | | |
| | 22 | Control | +++ | 580 | 0.69 | 26.6 | 2.73 |
| Fig. 1, A Tryptophane | 23 | Control | +++ | 1540 | 1.08 | 37.8 | 2.46 |
| Fig. 1, B | 24 | Control | ± | 900 | 1.04 | 24.9 | 4.27 |
| | 25 | Control | 0 | 1020 | 0.88 | 22.8 | 3.28 |
| | 26 | Control | 0 | 1220 | 1.12 | 24.0 | 3.18 |
| | 27 | Control | 0 | 1310 | 1.09 | 22.0 | 10.2 |
| Nicotinamide | | | | | | | |
| | 28 | Control | 0 | | | | |
| | | | | Ave. 1130 | 1.03 | 101.2 | 50 |
| | 1 Mar. | Control | 0 | | | | |
| | 2 | Control | 0 | 960 | 1.01 | 132.0 | 50 |
| | 3 | Control | 0 | 800 | 0.87 | 37.7 | 12.0 |
| | 4 | Control | 0 | | | | |
| | | | | Ave. 1030 | 1.08 | 38.5 | 17.3 |
| | 5 | Control | 0 | | | | |
| | 6 | Control | 0 | 1660 | 1.28 | 40.9 | 13.5 |
| | 7 | Control | 0 | 620 | 1.12 | 21.8 | 34.6 |
| | 8 | Control | 0 | 1360 | 1.09 | 17.6 | 15.3 |
| | 9 | Control | 0 | 1460 | 1.08 | 16.5 | 11.6 |
| | 10 | Control | 0 | 1400 | 1.16 | 16.3 | 28.5 |

*Course:* He was put to bed and on February 20 the experimental low B-complex diet was started. On February 24 he was given tryptophane in nine 100 mg. doses* at hourly intervals. On the same day his tongue became much less swollen and much less red and by the next day it had acquired a bluish magenta tint. On the following morning the bright red component was gone but the magenta color persisted (see Fig. 1). On March 1 he was given 100 mg. of nicotinamide every hour for ten doses. On March 17 a regimen of brewers' yeast and high carbohydrate, high protein diet was begun. The neuromuscular signs gradually improved. He was discharged on April 26, able to walk well with no pain or tenderness, though with a suggestion of a steppage gait. The tongue was normal.

CASE 2.—C. L., a 31-year-old farmer, was admitted to the Medical Wards of University Hospitals on March 24, 1949, and was discharged on April 16, 1949. On admission his chief complaints were swelling of the abdomen, anorexia, nausea, and vomiting. He had had

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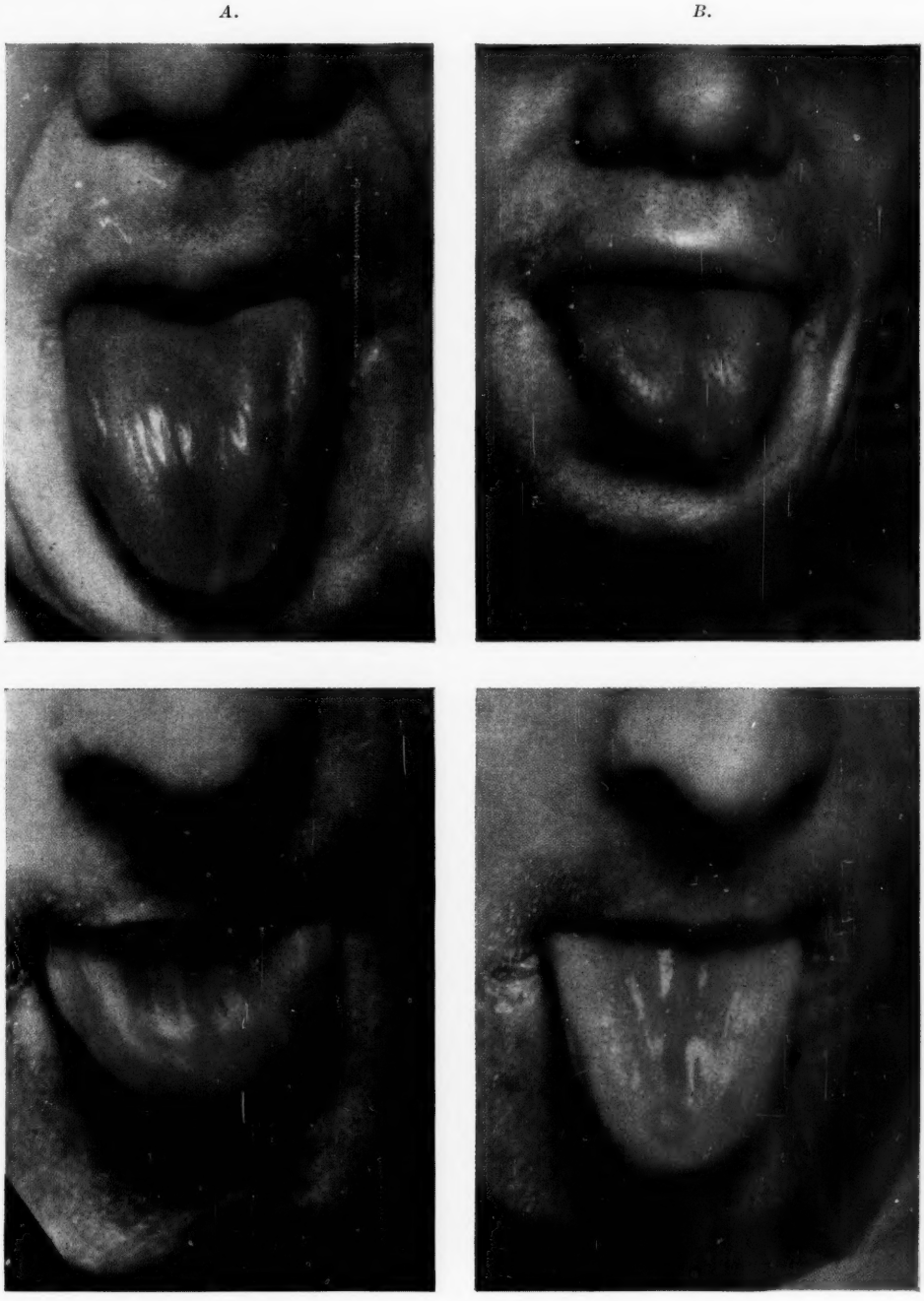


Fig. 1.—Acute pellagrous glossitis:

*A*, Subject E. K. (Case 1) before trytophane. Note also cheilosis.
*B*, Same subject twenty-four hours after therapy was begun.
*C*, Acute pellagrous glossitis. Subject C. L. (Case 2) before treatment.
*D*, Same subject twenty-four hours after instituting treatment with tryptophane.

scarlet fever at the age of 13 years with no evident sequelae. He was examined for induction into the Army in 1940; he was physically qualified but was deferred as a farm worker. Since 1945 he had drunk an excessive amount of alcohol including an occasional drink before breakfast. He had an adequate diet until August, 1947, when he lost his appetite, was troubled by frequent bouts of nausea with vomiting particularly following ingestion of fatty foods. He lost 30 pounds. Gastrointestinal and colon x-ray studies in March, 1948, had been normal. On a regimen of bland fat-poor diet there was symptomatic improvement. In June, 1948, he began to have swelling of the ankles and was forced to quit his farm work. He got a job in a machine factory but had to stop work altogether in November, 1948, because of progressing edema of the abdomen, legs, and feet. In December, 1948, and January, 1949, he had occasional chills and fever. He was put to bed at home and treated with penicillin injections. Abdominal paracenteses were done, two early in February and one in March, with removal of about 15 liters of fluid each time. There was no history of jaundice, hematemesis, melena, or hemorrhoids.

The patient was a pale, thin, white man with enormously distended abdomen. The sclerae were injected. There were extensive telangiectases over the cheeks and bridge of the nose, and many vascular spiders in the suprasternal notch and on the dorsum of the hands and arms. There was moderate palmar erythema and early clubbing of the fingers. The tongue was smooth and red. (See Fig. 1, *C*.) There was marked cheilosis. Hair was scanty in the axillae and absent from the chest, but there was no gynecomastia. The diaphragms were high to percussion and moved little with respirations. The abdomen was much distended with fluid, but there was no significant dilatation of the abdominal veins. After the removal of 8,000 c.c. of straw-colored fluid by abdominal paracentesis the sharp liver edge could be felt 6 cm. below the xiphoid process, the upper border of dullness being in the 4th intercostal space on the right. The tip of the spleen could be felt during deep inspiration. Rectal examination was negative. There was slight scrotal edema and advanced pitting edema of the lower extremities.

The patient was put to bed and given a diet high in carbohydrate and protein supplemented with brewers' yeast. He refused much of the food and yeast. Salt was restricted and mercurial diuretics were given frequently. There was rapid reaccumulation of fluid with abdominal pain and distention accompanied by a fever, the cause of which was not determined. Penicillin and streptomycin were given and the patient became afebrile.

*Course:* On March 29 he was given the control diet, having taken little of the diet or yeast. By April 2 the tongue was bright red and smooth. Tryptophane, in nine doses of 100 mg. at hourly intervals, was given on April 2. Before treatment the twenty-four hour urine contained 40.3 $\mu$g of thiamine and 12.5 mg. of $N^1$-methylnicotinamide. Two days later the thiamine was more than 50 $\mu$g and the niacin 33.4 milligrams. The tongue improved. (See Fig. 1, *C* and *D*.) Therapy with an ample diet was resumed on April 5. Abdominal paracentesis yielded 5,500 c.c. on April 6, 1949. The patient was discharged on April 16 with advice to stop drinking but to continue the diet and mercurial diuretics. He was seen again on May 23, 1949, in the Outpatient Clinic at which time he felt well. He had been able to resume light work. There was no edema or ascites; the liver border was 2 cm. below the costal margin but the spleen was not palpable. On Aug. 31, 1949, he was still better. He had some spider nevi though a few had disappeared and others were smaller, and the palmar erythema had almost disappeared. The liver was barely palpable below the costal margin but the spleen was not palpable. There was no edema or ascites and he appeared well nourished.

The laboratory data are summarized in Table II.

*Procedures.*—The patients remained in bed during the period of observation. The control diet contained 12.5 mg. of nicotinic acid, 65 to 70 Gm. of protein, 200 $\mu$g of thiamine, and 2,500 calories.[7] Urine was collected in twenty-four hour periods in bottles containing glacial acetic acid as a preservative. Before storage in the refrigerator the pH was adjusted to 4.0 to 5.0. As a check on the accuracy of collections creatinine was determined by the

*This unusual number was fortuitous. It was all we had on hand and we repeated the same dose schedule in Case 2.

TABLE II. CASE 2, C. L.

| | MARCH 24, 1949 | MAY 23, 1949 | AUG. 31, 1949 |
|---|---|---|---|
| R.B.C. | 4.51 | ---- | 3.78 |
| Hgb. | 14.0 | 12.1 | 13.0 |
| W.B.C. | 18,350 | 8,700 | 8,500 |
| Protein, total (Gm./100 ml.) | 5.36 | 6.80 | ---- |
| Albumin (Gm./100 ml.) | 2.68 | 3.86 | ---- |
| Globulin (Gm./100 ml.) | 2.68 | 2.94 | ---- |
| Urine bile | Trace | ---- | ---- |
| Urine urobilinogen (mg./liter) | 3.2 | ---- | ---- |
| Van den Bergh (mg./100 ml.) | Less than 0.5 mg. | Less than 0.5 mg. | ---- |
| Alk. phosphatase (Bodansky units) | 3.2 | ---- | ---- |
| Ceph. flocc. | 2+ in 48 hr. | 2+ in 48 hr. | ---- |
| Thymol turbidity (units) | 4.8 | 4.4 | ---- |
| Zn sulfate turbidity (units) | 14 | 13.9 | ---- |
| Cholesterol (mg./100 ml.) | 146 mg. | 180 mg. | ---- |
| Cholesterol esters (mg./100 ml.) | 89 mg. | ---- | ---- |
| Prothrombin time (sec.) | 35 | 42 | ---- |
| Prothrombin time control (sec.) | 30.3 | 35 | ---- |
| X-rays: | Normal esophagram. Chest—Elevation of both leaves of diaphragm with basilar atelectasis on the left. Abdomen (flat plate)—ascites, no evident adhesions | | |

Folin microchemical method[8] using a photoelectric colorimeter for reading. Thiamine was determined by the thiochrome method.[9, 10] $N^1$-methylnicotinamide was measured by the fluorometric method of Huff and Perlzweig[11] based on the original method of Najjar and Wood.[12] The remaining studies of liver function, carbohydrate tolerance, and blood were done with standard techniques.

## RESULTS

In contrast to the patients with pellagra with glossitis whose response was rather slow,[2] the two patients we are reporting had a rapid improvement in glossitis (see Fig. 1). A change was observed toward the end of the first day of therapy and had become substantially complete within twenty-four hours of giving the first dose of 100 mg. of tryptophane. The fiery red color was reduced in intensity. Edema decreased. In one instance a magenta color became evident. We suspect that fading of the erythema revealed it rather than that it was a new development because we have not seen such an acute appearance of the magenta color in a previously normal-looking tongue. There was very slight pain or burning but the complaint of slight irritation associated with eating or drinking disappeared with the color change. There were no other signs of pellagra suitable for judging therapy.

Laboratory studies of the urine reveal inconsistent results. In Case 1 there was a low excretion of $N^1$-methylnicotinamide while on the control diet, a distinct though small rise in excretion following therapy with tryptophane,

and a very large increase when the same dose pattern was repeated using nicotinamide in nine hourly doses of 100 mg. each. Associated with this large excretion of $N^1$-methylnicotinamide, and without change in diet, there was a fourfold increase in thiamine excretion, sustained for seventy-two hours. Thiamine excretion then declined and reached levels slightly lower than the control levels. Although excretion of $N^1$-methylnicotinamide declined after seventy-two hours, the level remained persistently higher than the early control level. Data from studies on Case 2 are inadequate for detailed analysis. The control level of $N^1$-methylnicotinamide was 12.5 mg. for twenty-four hours, or approximately four times that of Case 1 though there was no apparent difference in the severity of the glossitis. This emphasizes the well-known discrepancy between clinical signs of deficiency and chemical studies of vitamin excretion, for which a logical explanation has never been put forward. There was some increase in thiamine excretion.

These studies confirm the observations of Mueller, Vilter, and Bean on the efficacy of tryptophane in relieving the glossitis of human pellagra. While the quantity used was relatively small, tryptophane was effective in less than gram quantities under the circumstances of this experiment. The degree of tissue depletion was thought to be less extreme than in the patients reported previously.[2] Our control diet contained three times as much niacin as did the diet used in the other study.[2] Thus with a fair intake, and presumably only moderate tissue unsaturation, a relatively small dose of tryptophane was effective in producing a rapid clinical response.

A rise in excretion of end products of niacin metabolism occurred in both patients after tryptophane was administered. The differences in base line level and post-treatment levels are so divergent that their significance is obscure. We note but cannot explain the increased excretion of thiamine in association with the response to tryptophane therapy.

## CONCLUSION

Nine doses of tryptophane in divided hourly doses of 100 mg. each produced rapid improvement in the tongue of two patients with pellagrous glossitis who had not improved when fed a control diet with little niacin restriction.

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# ANEMIA IN ACUTE EXPERIMENTAL BERYLLIUM POISONING

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With the Technical Assistance of R. E. Root

ANEMIA has been a constant finding in dogs, rabbits, and rats inhaling beryllium salts at concentrations that produce few or no other toxic symptoms.[1] This paper describes the character of this anemia and of associated blood cellular changes, its indicated mechanism of production, the effects among different animal species following the administration of factors for the regeneration of the anemia (Lextron, folic acid, and vitamin $B_{12}$), and lastly, the nature of the recovery from the anemia.

## EXPERIMENTAL

Three rabbits confined in individual cages were exposed in an inhalation chamber to an aqueous aerosol of beryllium fluoride for six-hour periods daily, five days each week at a concentration of 2.2 ± 0.25 mg. per cubic meter of air. Exposure to the aerosol was continued for twenty-three weeks. The rabbits were fed a diet of Purina rabbit chow Checkers and oats supplemented with carrots twice weekly; food and water were withheld during exposure.

*Hematologic Procedures.*—Complete blood counts were made at weekly or biweekly intervals during exposure and during two weeks prior to exposure. The blood picture studied consisted of: red blood cell counts done in quadruplicate; white blood cell count and differential white blood cell count of at least 200 white cells; hemoglobin by the acid hematin method; hematocrit by the Wintrobe method; platelet count by a modification of the method of Rees and Ecker; and reticulocyte count by direct smear method.* The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were determined with each count. Differential counts of bone marrow samples were made from material taken by puncture through the crest of the ileum (in dogs only) at from two- to four-month intervals.

## RESULTS

*Character of the Anemia—Rabbits.*—Following exposure to the beryllium fluoride aerosol, the mean red blood cell count, initially $5.5 \times 10^6$ cells per cubic millimeter, began to decrease after two weeks and continued from the sixteenth to the twentieth week when the count registered $3.8 \times 10^6$; at the twenty-third week it was $4.3 \times 10^6$. As shown in Fig. 1, the mean corpuscular volume began to increase at the same time from a normal of 72 $\mu^3$ and continued to show a progressive and sharper increase as the red blood cell count decreased, reaching a maximum of 100 $\mu^3$ at the sixteenth week. There was a concomitant decrease in hemoglobin concentration of the whole blood, but this was followed

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*These methods are described by Kolmer and Boerner, "Applied Laboratory Technique," ed. 3, New York, 1941, D. Appleton-Century Company, Inc.

after the twelfth week of exposure by a return toward normal values. The mean corpuscular hemoglobin concentration varied within the normal range for rabbits, 33 ± 1.7 per cent. No changes either in white blood cell counts or differential counts were observed.

A pattern of hematologic response similar to that described for beryllium fluoride was obtained in another group of three rabbits exposed to beryllium oxide dust as shown in a comparative table (Table I).

TABLE I. HEMATOLOGIC RESPONSE OF RABBITS (MEAN AND MEAN RANGE OF 3 ANIMALS PER COMPOUND)

| | $BeF_2$ (2.2 MG./M.$^3$) | | BeO (80 MG. M.$^3$) | |
|---|---|---|---|---|
| | INITIAL | MAXIMAL ANEMIA | INITIAL | MAXIMAL ANEMIA |
| R.B.C. × $10^6$/mm.$^3$ | 5.5 ± 0.15 | 3.6 ± 0.2 | 6.0 ± 0.15 | 4.9 ± 0.2 |
| Hb. (Gm./100 ml.) | 12.4 ± 0.4 | 10.6 ± 0.3 | 12.1 ± 0.6 | 11.1 ± 0.2 |
| MCV ($\mu^3$) | 72 ± 3.7 | 98 ± 3.5 | 68.5 ± 4.5 | 85.6 ± 4.4 |

Hematologic response from the inhalation of beryllium, therefore, appears to resemble a macrocytic type of anemia in rabbits.

*The Effect of Dietary Supplements on Beryllium-Produced Anemia.*—In the following experiments certain dietary supplements commonly found beneficial in other types of anemia were tested to determine their effects on this toxic anemia produced by beryllium.

The experiments to be described use singly, Lextron[2]* folic acid,† and vitamin $B_{12}$‡ chiefly in dogs, but folic acid was used also in rats made anemic by exposure to beryllium. Both prophylactic and therapeutic tests were made with these agents.

*Lextron Supplement in $BeF_2$-Exposed Dogs.*—Prior to the start of the experiment, enteric parasitic infestations were reduced by a suitable deworming agent.§ One month before exposure, Lextron was added daily to the diet of six young, adult dogs (weighing on the average 6 to 8 kilograms) in amounts of 1 tsp. per day to the diet of each dog which consisted of 250 to 300 grams dry weight of Purina dog chow meal containing 10 per cent of Evr Redy bone meal made into a wet mash. Four dogs were maintained on the Lextron-supplemented diet during exposure while Lextron was denied the two remaining dogs which served as beryllium-exposed controls. Fig. 2 shows that a trend toward macrocytic anemia developed within two weeks of exposure (MCV values) which became maximal at the twenty-third week in the Lextron-fed dogs (MCV, 120 $\mu^3$; R.B.C. 3.3 × $10^6$ cells per cubic millimeter). The striking fact to observe is that anemia occurred similarly in the dogs fed Lextron and in the control dogs not fed Lextron; moreover, the degree of anemia was more marked in the Lextron-treated dogs as shown in Fig. 2. In the controls, the MCV at no time during

*A liver-stomach concentrate containing ferrous iron and vitamin B complex, generously contributed by Eli Lilly & Company, Indianapolis, Ind.

†Folic acid (Folvite), 90 per cent pure, generously contributed by Lederle Laboratories, Pearl River, N. Y.

‡Vitamin $B_{12}$ (Rubramine); a concentrate containing 30 $\mu$g per milliliter produced by E. R. Squibb & Sons, New York, N. Y.

§Canine Crystoids, Sharpe & Dohme, Inc., Philadelphia, Pa.

the thirty-two weeks of exposure reached values as high as 110 $\mu^3$ nor did the R.B.C. drop to values lower than $4 \times 10^6$ per cubic millimeter. Similarly striking was the finding that the only two dogs that died during exposure to the aerosol were those two in the group receiving the Lextron-supplemented diet, one of which died at four and one at ten weeks of exposure.*

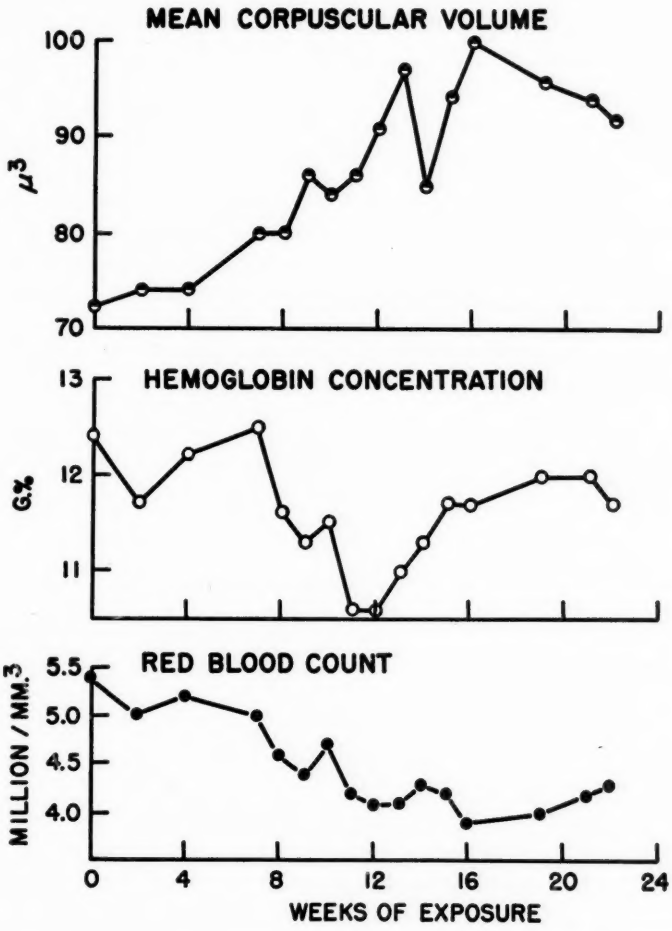


Fig. 1.—Blood changes in rabbits following exposure to beryllium fluoride mist.

*Changes in Reticulocytes.*—Reticulocyte counts in three of the dogs determined by both the standard method and the Nizet technique[5] decreased steadily during the first twelve weeks of exposure and thereafter tended to remain low. A fourth dog showed a reticulocytosis, normoblasts appearing in the peripheral blood which increased to a peak of 28 per 100 leucocytes during the sixteenth week of exposure, after which they declined in frequency and disappeared. At the same time, erythroblasts occurred in the circulating blood, increasing from 1 to 4 per cent (erythroblasts) per 100 leucocytes during the fourth month of

*Similar results occurred with folic acid reported below.

exposure and persisting thereafter. This occurred only in the dog exhibiting the greatest degree of anemia of the group (R.B.C. count 3.3 × $10^6$ per cubic millimeter; hemoglobin, 3.9 Gm. per 100 ml.).

*Leucocyte Changes.*—An increase in the percentage of nonsegmented (immature) polymorphonuclear neutrophil granulocytes occurred in all groups of beryllium-exposed dogs which was less marked in the dogs not fed Lextron. Atypical, "foamy" lymphocytes were seen in blood smears in both groups of dogs during the second to the sixth month of exposure. Their significance is unknown.

*Bone Marrow Changes.*—Specimens biopsied from the crest of the ileum of dogs at eighteen and thirty-two weeks indicated increased erythropoiesis.

*Folic Acid Supplement in Beryllium Sulfate-Exposed Dogs and Rats.*—Eight dogs and forty rats were divided into equal groups of four and twenty respectively. One group of each species was placed on a control diet which in the case of the dog was the same as already described and for the rats consisted of 20 Gm. per day per rat of Purina fox chow meal. The diets of the other group were supplemented daily with folic acid. Dogs were given folic acid in amounts of 10 mg. per day in two divided doses by capsule orally. The rats received folic acid in amounts of approximately 2 mg. per day per rat on the basis of food consumed (2.5 mg. folic acid per 20 Gm. diet). Weekly blood counts were made in quadruplicate on the dogs; on the rats, after four and six weeks only.

TABLE II. HEMATOLOGIC RESPONSES IN DOGS EXPOSED TO BERYLLIUM SULFATE MIST AT 4 MG./M.$^3$

| | RED BLOOD COUNT X $10^6$ | | HEMOGLOBIN (GM. %) | | MEAN CORPUSCULAR VOLUME ($\mu^3$) | |
|---|---|---|---|---|---|---|
| DOG | BEFORE EXP. | AFTER 6 WK. EXP. | BEFORE EXP. | AFTER 6 WK. EXP. | BEFORE EXP. | AFTER 6 WK. EXP. |
| | | | *Folic-Acid Treated* | | | |
| 1407 | 7.0 | 5.5 | 15 | 13 | 70 | 80 |
| 1434 | 6.9 | 5.1 | 15 | 13.5 | 70 | 86 |
| 1416 | 6.9 | 4.8* | 15 | 11 | 72 | 94† |
| 1360 | 7.6 | 5.6* | 16 | 15 | 70 | 86† |
| Average | 7.1 | 5.2 | 15 | 13 | 70.5 | 86.5 |
| | | | *No Folic Acid* | | | |
| 1391 | 6.6 | 4.4 | 13 | 12 | 66 | 86 |
| 1401 | 7.2 | 5.5 | 16 | 14 | 70 | 86 |
| 1405 | 7.0 | 5.5 | 14 | 12 | 66 | 85 |
| 1406 | 7.2 | 5.4 | 15.5 | 14.5 | 70 | 86 |
| Average | 7.0 | 5.2 | 14.6 | 13.1 | 68 | 86 |

*Death occurred in these animals: Dog 1360 after three weeks' exposure; dog 1416 after six weeks' exposure.

†Highest value attained.

*Dogs:* Table II shows the individual and mean changes in the red blood cell picture at six weeks of exposure, viz., a decrease of 1.9 × $10^6$ cells per cubic millimeter, of 2 Gm. hemoglobin, and a rise of 17 $\mu^3$ in cell volume. No significant differences occurred in the averages of any of the variables of either experimental group.

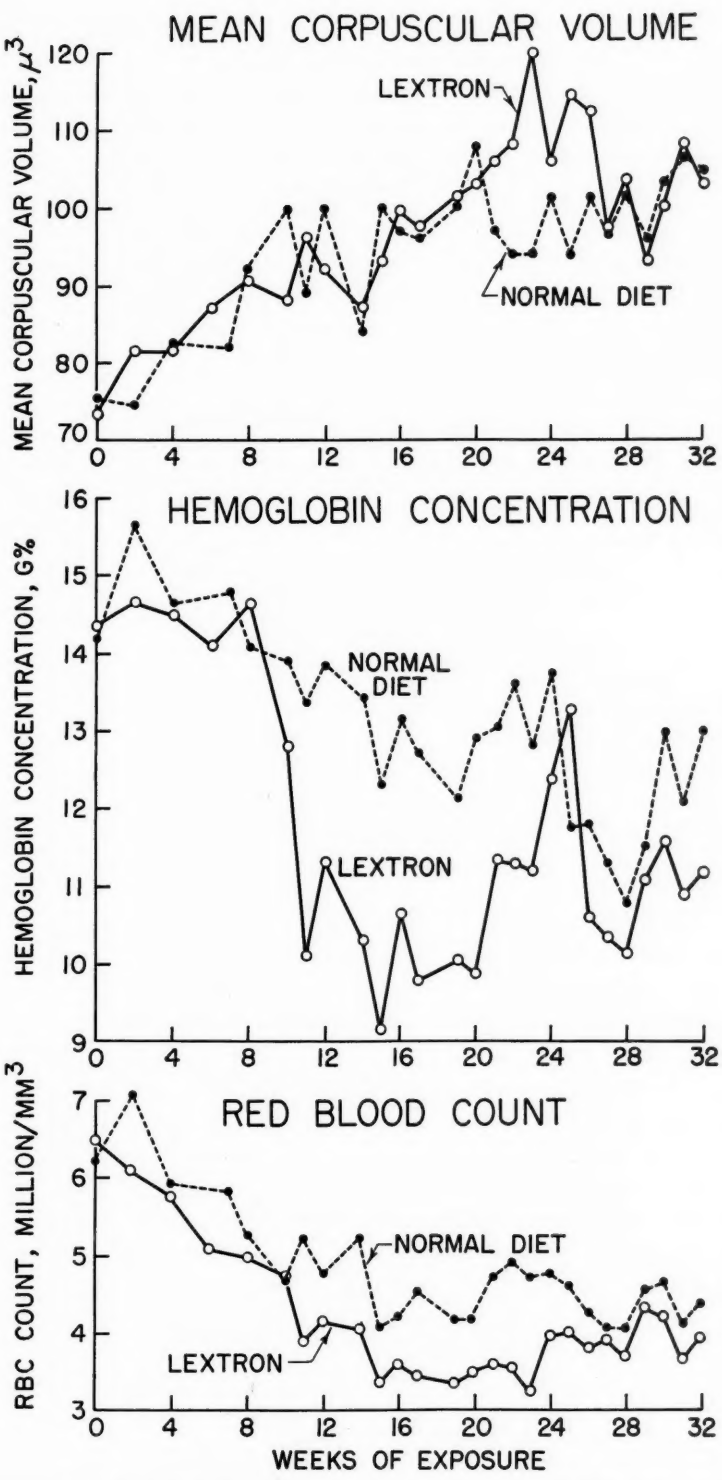


Fig. 2.—Blood changes in dogs exposed to beryllium fluoride mist.

*Rats:* In contrast to the dogs in which folic acid was not only without benefit but appeared to act unfavorably, folic acid in rats prevented the development of macrocytic anemia. The beryllium-induced anemia in rats not given folic acid (Table III) differs from that in dogs in that the hemoglobin level of the whole blood is not altered, while changes occur in the R.B.C. count and the mean corpuscular hemoglobin. In the rats receiving no folic acid, the average R.B.C. count fell to $1.9 \times 10^6$ cells per cubic millimeter and the MCH rose 13 $\mu\mu$g, whereas no appreciable changes occurred in these components in the folic acid-treated rats. It appears, therefore, from Table III, that folic acid in amounts of approximately 2 mg. per day per rat is effective in preventing the development of the macrocytic anemia.

TABLE III. HEMATOLOGIC CHANGES IN RATS WITH AND WITHOUT FOLIC ACID AND EXPOSED DAILY TO BERYLLIUM SULFATE MIST

| | AFTER 6 WEEKS OF EXPOSURE | | |
|---|---|---|---|
| RAT | R.B.C. x $10^6$ MM.$^3$ | Hb. (GM. %) | MCH ($\mu\mu$g) |
| | *Folic Acid Treated* | | |
| 8 | 6.9 | 18.7 | 27 |
| 9 | 8.0 | 15.8 | 20 |
| 10 | 7.7 | 14.6 | 19 |
| 12 | 7.2 | 16.0 | 23 |
| 14 | 7.0 | 16.9 | 24 |
| Average | 7.4 | 16.4 | 24 |
| | *Controls—No Folic Acid* | | |
| 26 | 5.3 | 15.6 | 30 |
| 33 | 5.7 | 16.2 | 29 |
| 34 | 5.6 | 21.1 | 38 |
| 36 | 5.5 | 17.9 | 33 |
| 39 | 5.2 | 15.0 | 29 |
| Average | 5.5 | 17.1 | 33 |

*Effect of Vitamin $B_{12}$—Dogs.—*

*Prophylactic Use:* Four dogs were exposed to beryllium fluoride mist at a level of 2.2 mg. per cubic meter under conditions already described, with the object of evaluating the prophylactic effectiveness of this vitamin against the anemia. Two of these dogs received intramuscularly a dose of 3 $\mu$g vitamin $B_{12}$ three times during the first week of exposure at forty-eight hour intervals and thereafter twice weekly at intervals of seventy-two hours during the seven weeks of the experiment; the other two dogs served as controls receiving no vitamin $B_{12}$ but receiving the same exposure to beryllium fluoride. In both groups, the R.B.C. counts decreased as exposure progressed as did the hemoglobin concentrations of the whole blood, but the average depression of the R.B.C. counts was somewhat less in the vitamin $B_{12}$ dogs than in the controls (0.75 as against $1.3 \times 10^6$ cells per cubic millimeter). On the other hand, there was no significant difference between the decreased hemoglobin concentrations of the two groups nor was there any trend in the leucocytic and differential counts of these dogs. From these results, it would appear that vitamin $B_{12}$ alone is of little prophylactic value in the prevention of this type of anemia induced by exposure to beryllium fluoride.

TABLE IV. BERYLLIUM CONTENT OF TISSUES AT TIME OF RECOVERY FROM ANEMIA FOLLOWING INHALATION OF BERYLLIUM SULFATE

| DOG | TIME OF EXPOSURE BEFORE REMOVAL FROM CHAMBER (DAYS) | TIME OF DEVELOPMENT OF ANEMIA* (DAYS) | DEGREE OF ANEMIA DECREASE IN | | TIME OF RECOVERY FROM ANEMIA (DAYS) | TIME FROM END OF RECOVERY TO SACRIFICE (DAYS) | Be CONTENT OF TISSUES (μg/GM. FRESH TISSUE) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | R.B.C. ($\times 10^6$ MM.$^3$) | Hb. (GM. %) | | | LUNG | BONE | LIVER | KIDNEY |
| 1432 | 70 | 66 | 2.0 | 6.6 | 104 | 0 | 2.9 | 1.3 | 0.5 | 0.1 |
| 1418 | 65 | 70 | 2.4 | 7.4 | 115 | 21 | 1.2 | 1.2 | 0.4 | 0.02 |
| 1425 | 64 | 55 | 1.7 | 3.4 | 115 | 21 | 0.5 | 0.4 | 0.1 | 0.002 |

*Time of greatest decrease in R.B.C. count and hemoglobin concentration.

*Therapeutic Use:* In another experiment, two dogs that had been exposed for twenty-eight weeks to beryllium fluoride mist and that had developed the macrocytic type of anemia were given 12 $\mu$g each of vitamin $B_{12}$ intramuscularly in four divided doses at seventy-two hour intervals while daily exposure continued. One of these dogs had been receiving Lextron but the other had been given an unsupplemented diet during the previous twenty-eight weeks of exposure. Both dogs showed a slight to moderate reticulocytosis followed by an increase in the R.B.C. count of one million cells. This increase was not sustained and it was concluded that vitamin $B_{12}$ is not an effective therapeutic agent in dogs under these conditions. A similar response to $B_{12}$ had been observed previously in dogs made anemic by beryllium sulfate mist.

*Recovery From Beryllium-Induced Anemia.*—Four dogs were exposed to beryllium sulfate mist for the threefold purpose of determining whether recovery occurs from the toxic anemia produced by the inhalation of beryllium salts, and, if so, the time required, and whether recovery is compatible with the presence of beryllium in the tissues. If the last is established, it would indicate that a tissue tolerance to beryllium occurs in animals.

Four growing dogs, weight ranging from 5.6 to 9.7 kilograms, were exposed to beryllium sulfate mist at a concentration of 3.6 mg. per cubic meter of air, six hours daily until the toxic anemia developed. Anemia was considered established when a decrease of 2 million red blood cells per cubic millimeter and a decrease of at least 3 Gm. per cent in hemoglobin concentration occurred. Exposure was forthwith terminated and the animals observed for recovery. During this period, as well as during and prior to exposure for three weeks, complete blood counts were made weekly. Upon recovery, animals were sacrificed and the tissues analyzed* for beryllium content spectrographically by methods developed by Steadman.[3] The samples of femur for analysis comprise the head and 1 in. of the shaft.

It is seen in Table IV that anemia developed after about two months of daily exposure (55 to 70 days) and that 104 to 115 days were required to restore a normal blood picture (6.6 to 7 million red blood cells per cubic millimeter; hemoglobin 14 to 16.3 Gm. per cent). Following recovery these dogs were sacrificed and beryllium was found in appreciable quantities in all four tissues analyzed—lung, femur, liver, and kidney (Table IV).

## DISCUSSION

The anemia that developed in animals from the inhalation of beryllium salts was mild in degree and did not result in death. It was considered macrocytic in type in that the mean red cell volume enlarged to values of from 100 to 120 $\mu^3$, while the red cell count fell from 2 to 3 million cells per cubic millimeter. This anemia, however, has three aspects that make it of more than usual interest. One is the different character of the anemia among species. In the dog, the R.B.C. count, MCV, and hemoglobin all change in a manner typical of a normochromic marcocytic anemia; in the rabbit there is a lessened tendency for the hemoglobin concentration of the whole blood to decrease and a tendency to re-

*Analysis performed through courtesy of Dr. L. T. Steadman.

turn to normal values is more pronounced; in the rat the hemoglobin values are normal while the two other variables change in a manner consistent with a macrocytic anemia.

The second aspect of unusual nature is the species specific response to the treatment with the blood-stimulating agents Lextron, folic acid, and vitamin $B_{12}$. In dogs, Lextron and folic acid failed to benefit the anemia and appeared to contribute to the fatal outcome of certain of these animals. In another species, the rat, folic acid reversed the pattern of action found in dogs and not only prevented the development of the anemia under the same conditions of exposure but appeared to benefit the species generally from the effects of beryllium poisoning.

A possible explanation for the detrimental effect of Lextron on the anemia in dogs might be that its iron, a known gastric irritant, further added to the irritant effect of beryllium brought to the gastrointestinal tract by ciliary action in the respiratory system. Whether a similar explanation holds for folic acid is not known.

The third aspect of interest centers on the mode of development of anemia by beryllium. The complete mechanism is by no means clear, although the following pertinent facts have been noted in the course of the work.

1. Little or no evidence could be obtained for the view that the anemia was hemolytic in character. A normal icteric index was found in animals (dogs) as well as normal plasma bilirubin values with the exception of an occasional value double the normal range. In the dogs showing these occasional rises, there was a somewhat increased retention of bilirubin as shown by clearance tests. Moreover, it was not possible to demonstrate excess output of urinary porphyrins or presence of unusual types in rabbits by the method of Dobriner and Rhoads.[4]

2. The possibility that the anemia was not of sufficient degree to provide adequate stimulus for additional erythropoiesis is shown by the reticulocyte counts which instead of increasing, decreased. Moreover, bone marrow smear examination showed only slight evidence of increased erythropoiesis in some animals but in others was normal.

3. Much discussion has centered on the presence or absence of beryllium in tissues in relation to the disease processes. The development of anemia following the deposition of beryllium in the tissues would indicate a causal relationship between beryllium and anemia, but the finding of beryllium near the site of erythropoiesis during and after recovery from the anemia would indicate the ability of the tissue to develop a tolerance to beryllium.

The development of the same type and degree of anemia by three compounds of beryllium containing different anions, sulfate, oxide, and fluoride, indicates that beryllium is the causative agent. As to the question of the possible role of the other anions, only fluoride would be of concern. Evidence that fluoride plays no part in this anemia is afforded by hematologic studies on the same three species of animals, the dog, rabbit, and rat, during thirty-day exposures by inhalation to hydrogen fluoride at 25 mg. per cubic meter.[6] Hemo-

grams of twenty variables showed no changes of consequence in the three species at a concentration that was toxic to the dog and rabbit and lethal to the rat. Moreover, evidence from a hematologic study of rats ingesting 50 ppm sodium fluoride in the drinking water showed the fluoride ion to be without effect on either the hemoglobin or on the hematocrit.[7] Concentrations of fluoride in both these studies were far in excess of those used in the present study.

## SUMMARY AND CONCLUSIONS

Anemia has been produced in three species of animals, the dog, rat, and rabbit, inhaling beryllium salts. The anemia resembles the macrocytic type, is mild in degree, and differs according to species.

Attempts at treating the anemia with common blood-regenerating agents appeared to be successful, as was prophylaxis, in limited numbers of one species (rat) whereas the treatment worsened another (dogs).

Recovery from the anemia occurred spontaneously, requiring from 3.5 to 4 months in dogs, despite the presence of considerable amounts of beryllium in the tissues.

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# EFFECT OF INTRAVENOUS FOLIC ACID ON THE CHOLINESTERASE ACTIVITY OF THE BLOOD AND THE FOLIC ACID BLOOD LEVELS

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RECENTLY attention has been directed toward the possible relationship between folic acid or liver extract and the changes in erythrocyte and plasma cholinesterase activity in pernicious anemia during therapy with these substances.[1-4] Davis and co-workers[5-8] have reported that liver extract and folic acid probably are effective in the treatment of pernicious anemia because of their capacity for increasing serum cholinesterase activity both in vitro and in vivo. However, Grob and co-workers[2] and Kunkel and associates[9] have presented evidence which suggests that the increases in erythrocyte or plasma cholinesterase activity, which occur in the blood of patients ill with macrocytic anemia responding to therapy with folic acid, are the result of the erythropoietic activity of the bone marrow and not the cause of it. In view of these differences in results, it seemed advisable to study this problem further. The purpose of this investigation was to determine the effect of the intravenous injection of folic acid both on the erythrocyte and plasma cholinesterase activity and on the folic acid blood levels in man.

## METHODS

Folic acid (pteroylglutamic acid) was injected intravenously into ten individuals, while fasting, in amounts of either 3.75 or 15.0 mg. The individuals studied included the following: three healthy males, three male patients ill with hepatic cirrhosis, one male patient ill with essential hypertension, and three patients ill with pernicious anemia, one during remission, the other two after a reticulocyte response to liver therapy. In some individuals separate observations were made following the administration of each of the two different amounts of folic acid. Plasma and erythrocyte cholinesterase activities and folic acid blood levels were determined before and at periodic intervals following the administration of the folic acid. The first specimen usually was obtained one-half hour after the injection, and the rest of the specimens at hourly intervals. Venous blood was collected in heparinized tubes for use in the various determinations.

The plasma and erythrocyte cholinesterase activities were determined by the electrometric method of Michel,[10] as modified by Scudamore and his associates,[4] using acetylcholine bromide as a substrate and expressing the results as the decrease in pH per hour (Δ pH per hour). The range of erythrocyte cholinesterase values for healthy individuals varies from 0.49 to 0.93 Δ pH per hour; the plasma cholinesterase values, from 0.62 to 1.30 Δ pH per hour. The folic acid content of the blood was measured by the bio-assay method of Tepley and Elvehjem[11] using *Lactobacillus casei* as the test organism. The blood folic acid levels were recorded as millimicrograms per milliliter.

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## RESULTS

A summary of the results of fourteen tests on ten individuals is shown in Table I, and three typical studies are illustrated in Fig. 1. The initial erythrocyte cholinesterase activity varied from 0.57 to 0.86Δ pH per hour, with a mean value of 0.66Δ pH per hour; the plasma cholinesterase activity, from 0.13 to 0.93Δ pH per hour, with a mean of 0.62Δ pH per hour. In none of the individuals studied was there any significant increase in either the plasma or the erythrocyte cholinesterase activity up to five hours after the intravenous administration of either 3.75 or 15.0 mg. of folic acid. In two tests no changes in cholinesterase activity were noted twenty-four to forty-eight hours after the injections. In four instances temporary decreases in plasma cholinesterase

TABLE I. AVERAGE CHANGES IN CHOLINESTERASE ACTIVITY OF THE BLOOD AND IN FOLIC ACID BLOOD LEVELS FOLLOWING INTRAVENOUS INJECTION OF FOLIC ACID* INTO TEN SUBJECTS

| TIME (HOURS) | CHOLINESTERASE ACTIVITY (Δ pH/HR.) | | FOLIC ACID BLOOD LEVELS (MMCG./ML.)† |
|---|---|---|---|
| | ERYTHROCYTE | PLASMA | |
| 0 | 0.66 | 0.62 | 55.3 |
| 1 | ---‡ | ---‡ | 294.7 |
| 3 | 0.66 | 0.61 | 145.0 |
| 5 | 0.66 | 0.61 | 123.0 |

*Folic acid, 15.0 or 3.75 mg., injected at zero hours.
†mmcg. = millimicrograms.
‡Determinations were not made in all instances at one hour.

activity, up to 10 per cent, were observed about one hour after the folic acid was given. The erythrocyte cholinesterase activity did not decrease significantly in any of the subjects.

As shown in Fig. 1 and Table I, the maximum folic acid blood levels were obtained one-half to one hour after injection, and then the levels decreased. The fasting blood levels averaged 56 mmcg. per milliliter, and ranged from 8 to 131 mmcg. per milliliter. The highest fasting value recorded was in subject J. E. (Fig. 1), who was receiving injections of liver extract as therapy for pernicious anemia. The maximum blood levels of folic acid ranged from 130 to 313 mmcg. per milliliter one-half hour after the injection of 3.75 mg. of folic acid, and from 450 to 679 mmcg. per milliliter after 15.0 mg. of folic acid. The blood folic acid levels usually remained above the fasting levels five hours after its injection. No significant differences in the nature of the response to folic acid were noted in either the healthy individuals or in the patients.

## DISCUSSION

Substantial elevations of the folic acid levels of the blood were obtained by the intravenous administration of either 3.75 or 15.0 mg. of folic acid. The peak folic acid blood levels observed in this study were in the range of those reported by Schweigert and his co-workers[12] following the intravenous injection of 12.0 mg. of pteroylglutamic acid; the levels obtained with the 3.75 mg. dose were lower and those with 15.0 mg. higher. However, in most of our subjects the fasting blood levels of folic acid were somewhat higher than those reported by these investigators.

EFFECT OF INTRAVENOUS FOLIC ACID ON CHOLINESTERASE ACTIVITY AND FOLIC ACID BLOOD LEVELS

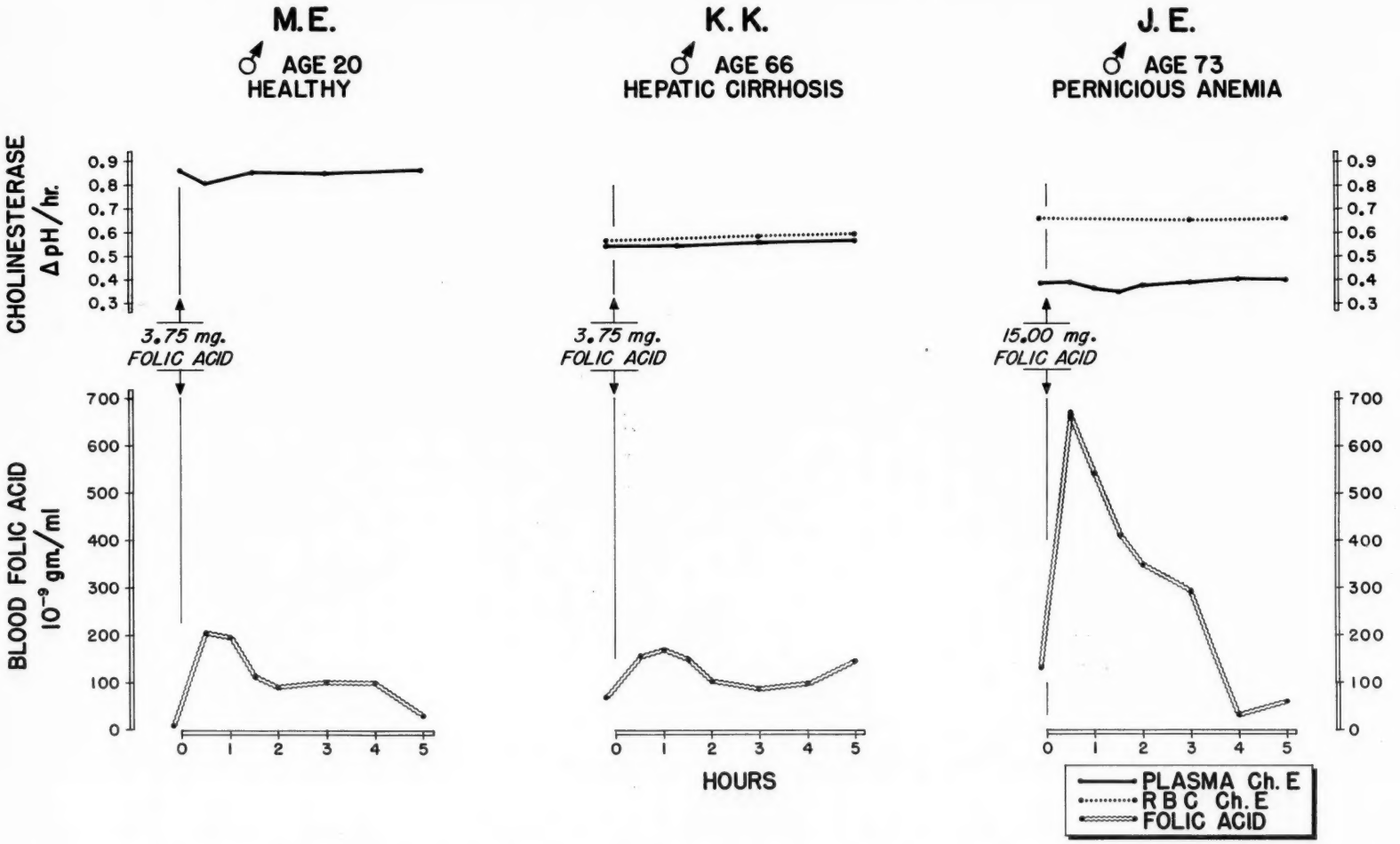


Fig. 1.—Effect of intravenous administration of folic acid on erythrocyte (R B C) and plasma cholinesterase (Ch. E) activity, and on folic acid blood levels in a healthy young man (M. E.), a patient ill with cirrhosis (K. K.), and another with pernicious anemia (J. E.).

Our results support the findings of Grob and associates,[2] and of Kunkel and co-workers,[9] that folic acid produces no immediate changes in the cholinesterase activity of the blood. The data presented here are at variance with the reports of Davis and co-workers,[5-8] who found that administration of folic acid increases the serum cholinesterase activity. Davis and Hamilton[8] reported that, following the oral administration of 15.0 mg. folic acid to nine normal human subjects, the serum cholinesterase activity increased from 14 to 33 per cent within five hours. In contrast, the experiments reported here revealed no significant changes in erythrocyte or plasma cholinesterase activity of the blood in healthy subjects or in patients ill with pernicious anemia or certain other diseases following intravenous folic acid.

Despite the apparent lack of an immediate effect of a single injection of folic acid on the blood cholinesterase activity, it has been demonstrated on patients suffering with pernicious anemia in relapse that prolonged therapy with sufficient quantities of folic acid, liver extract or vitamin $B_{12}$ will produce increases in erythrocyte cholinesterase activity.[1-4] The onset of the increase in erythrocyte cholinesterase activity usually coincided with the onset of the reticulocyte response and continued with active erythropoiesis. These same investigators noted that the low plasma cholinesterase activity, which is observed in most patients ill with pernicious anemia, improved more slowly than erythrocyte cholinesterase activity.

Thus, on the basis of this and other data,[4] it appears that the increases in erythrocyte and plasma cholinesterase activity of patients ill with pernicious anemia treated with folic acid or liver extract are brought about by erythropoiesis and recovery and not by the direct stimulation of cholinesterase activity by liver extract or folic acid. However, to definitely establish these relationships, the influence of intravenous folic acid on blood cholinesterase activity in subjects with a pre-existing deficiency of this vitamin should be determined. It is possible that a prompt change might be noted in the deficient subject instead of the delayed one associated with erythropoiesis.

## SUMMARY

1. The intravenous administration of either 3.75 or 15.0 mg. of folic acid does not alter significantly the plasma or erythrocyte cholinesterase activity in the blood of healthy subjects or of patients ill with cirrhosis, essential hypertension, or pernicious anemia during therapy.

2. The administration of these amounts of folic acid produces substantial elevations in the blood levels of folic acid which were at a maximum when first measured one-half to one hour after injection.

3. The relationship of these results to changes in erythrocyte and plasma cholinesterase activity observed in patients suffering with pernicious anemia during therapy with folic acid or liver extract is discussed.

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# THE EFFECT OF TRYPSIN ON THE DUFFY FACTOR

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THE specific antibody for the Duffy factor was discovered by Cutbush, Mollison, and Parkin[1] and designated anti-Fy$^a$. This factor determines two phenotypes, Duffy positive, Fy(a+), and Duffy negative, Fy(a–). Cutbush and Mollison[2] found that 64.88 per cent of individuals tested were positive and 35.2 per cent were negative, and Race, Holt, and Thompson[3] reported that 65.4 per cent were positive and 34.51 per cent were negative. In round figures, therefore, about two-thirds of random individuals in England are positive and one-third negative. Since the discovery of the Fy$^a$ factor, several post-transfusion hemolytic reactions due to it have been reported and therefore it is of some clinical as well as hemotologic significance.[4-7]

Race and Sanger[8] referring to anti-Fy$^a$, state that "the antibody is in the incomplete form. The interaction of antibody and antigen is made apparent only by the indirect antiglobulin test and not by the albumin nor the trypsin methods; in this way the antibody differs from the incomplete anti-Rh."

The present study was undertaken to explain the failure of trypsin-treated Fy(a+) cells to react with their specific antibody and to determine the results obtained when applying the trypsinated cell indirect antiglobulin method of titration.[9]

## MATERIALS AND METHODS

Two examples of anti-Fy$^a$ serum were used.† Both were specific and contained no other antibodies. Red blood cells known to be O N S $Rh_1rh$ P+ Lu(a–) Le(a+) Kk Fy(a+) were selected and used as Duffy positive cells. As a control, Duffy negative cells known to be O M S $Rh_2rh$ P+ Lu(a+) Le(a–) kk Fy(a–) were used.

These two specimens of cells were each divided into two parts. One part of each was washed four times in normal saline and a 5 per cent normal saline suspension was made. These were used as untreated Fy(a+) and untreated Fy(a–) cells. The other part of each specimen was treated with trypsin as follows. The cells were washed four times in normal saline solution and then packed. One part of a 1 per cent trypsin (Difco) in standard buffer solution was added to 9 parts of packed cells, placed in a water bath at 37° C. for one hour, and after one washing with normal saline, a 5 per cent saline suspension was made. These were used as trypsinated Fy(a+) and trypsinated Fy(a–) cells.

Titrations were carried out in the usual fashion. One drop each of progressively doubled dilutions of serum was put in a series of small test tubes (8 mm. in diameter). Then 1 drop of the 5 per cent test cell suspension was added to each tube. Incubation was carried out in a water bath at 37° C. for one hour. The sediment in the tube was then examined for clumping, under the microscope after removing the lower half of the low-power objective. For the albumin-plasma method, after the first hour of incubation the supernatant fluid was

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removed and replaced with a drop of a mixture of 4 parts of pooled oxalated plasma of Rh-positive individuals and 1 part of 30 per cent bovine albumin solution followed by an additional hour of incubation at 37° C. and then examined for clumping.

## RESULTS

Anti-Fy$^a$ serum was first titrated against cells known to be Fy(a+) and Fy(a–) (Table I). By the saline agglutination, the albumin-plasma, and the trypsinated cell methods no agglutination resulted with either untreated Duffy positive or untreated Duffy negative cells. However, by the antiglobulin method,* the titer was 16 units when tested against untreated Duffy positive cells and negative against untreated Duffy negative cells used as a control. These results are typical and conform to the criteria of Race and Sanger.

TABLE I. TITRATION OF ANTI-Fy$^a$ SERUM

| METHOD | TYPE CELL | DILUTION OF SERUM | | | | | |
|---|---|---|---|---|---|---|---|
| | | UNDIL. | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| Saline agglutination (untreated cells) | Fy(a+) | – | – | – | – | – | – |
| | Fy(a–) | – | – | – | – | – | – |
| Albumin-plasma (untreated cells) | Fy(a+) | – | – | – | – | – | – |
| | Fy(a–) | – | – | – | – | – | – |
| Trypsinated cells* | Fy(a+) | – | – | – | – | – | – |
| | Fy(a–) | – | – | – | – | – | – |
| Anti-human globulin (untreated cells) | Fy(a+) | ++ | ++ | ++ | + | ± | – |
| | Fy(a–) | – | – | – | – | – | – |

*Cells treated either with papain or ficin gave the same results.

The serum was next titrated by three methods (saline agglutination, albumin-plasma, and trypsinated cell methods), but in this experiment the anti-human globulin test was carried out on the cells used with each serum dilution of all three methods. This test, applied to the cells of the saline agglutination titration as well as those of the albumin-plasma titration† yielded a titer of 16 units for both methods as was to be expected. When, however, the antiglobulin test was applied to cells used with each serum dilution of the trypsinated cell titration the result was completely negative (Table II). This is at variance with results when the same technique is used with $Rh_0$-positive cells coated with univalent $Rh_0$ antibodies where higher titers are obtained with the trypsinated cell indirect antiglobulin test than by any other.[9] In addition, this method is able to detect $Rh_0$ univalent antibodies when other methods fail because the titer is below the threshold of those tests.

This finding with the Duffy factor, however, we have repeatedly confirmed and with two different examples of anti-Fy$^a$. Blood specimens of fifty-two random individuals were tested. Using known anti-Fy$^a$ serum, thirty-two or 61.6 per cent proved to be Duffy positive as determined by the indirect antiglobulin test and twenty or 38.4 per cent Duffy negative. However, when

*The cells are washed four times in normal saline solution and then packed. The saline is completely decanted. The packed cells are then mixed with 2 drops of anti-human globulin serum (rabbit). The mixture is centrifuged lightly (500 to 1,000 r.p.m.) for one minute and the sediment gently resuspended and examined for clumping.

†The albumin-plasma titration was included merely for the sake of completeness. The cells of this titration were washed six times instead of the usual three times to insure removal of all traces of albumin.

TABLE II. TITRATION OF ANTI-Fy$^a$ SERUM BY INDIRECT ANTI-HUMAN GLOBULIN TEST ON UNTREATED AND TRYPSINATED CELLS

| CELL SEDIMENT OBTAINED FROM TITRATIONS | DILUTION OF SERUM (TITER IN UNITS) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 16 | 32 | 64 |
| Saline agglutination method (untreated) | ++ | ++ | ++ | ++ | + | Tr. | – |
| Albumin-plasma method (untreated) | ++ | ++ | ++ | +± | ± | – | – |
| Trypsinated cell method | – | – | – | – | – | – | – |

identical cells were first trypsinated, all fifty-two specimens appeared to be Duffy negative. The routine of this latter test subjects the cells to one additional procedure as compared with the former test, namely addition of trypsin and incubation at 37° C. for one hour followed by washing with normal saline. To be certain that this inequality played no role, the untreated cells were handled in identical fashion, substituting saline for trypsin.

In order to test further the possibility that the effect of trypsinating Duffy positive cells might be mechanical in nature, they were subjected to twelve successive washings with normal saline. Indirect antiglobulin test then resulted in clumping of the same degree as with the standard routine.

Two other enzymes, papain and ficin, were next substituted for trypsin and each yielded the same results. When Fy(a+) cells were treated with either of these enzymes and then used as test cells to titrate serum known to contain anti-Fy$^a$, no clumping occurred. When an antiglobulin test was done on the cells used in these titrations, no clumping occurred in any tube, showing that Duffy antibodies were unable to react with Fy(a+) cells after they had been treated with papain or ficin as well as with trypsin.

To determine the reason anti-Fy$^a$ reacts with untreated Fy(a+) cells yet fails to clump identical cells when treated with enzyme, absorption experiments were carried out. The titer of this example of anti-Fy$^a$ was 32 units. A measured amount of this serum was put into each of four tubes. To each tube was added separately a volume of washed packed cells equal to one-half the volume of serum. However, in each tube a different variety of packed cells was used, namely, untreated Fy(a+), trypsinated Fy(a+), untreated Fy(a–), and trypsinated Fy(a–). The two sets of Fy(a+) cells came from one individual as did the two sets of Fy(a–) cells. All tubes were put in a water bath at 37° C. for one hour, centrifuged, the supernatants removed and titrated for anti-Fy$^a$. Table III gives the results. After this absorption, the titer of serum absorbed with untreated Fy(a+) cells was 4 units, whereas those absorbed with trypsinated Fy(a+), untreated Fy(a–), and trypsinated Fy(a–) cells were each 16 units; the reduction in titer of one tube as compared with the unabsorbed serum was due to the normal range of the test or to dilution resulting from the absorption process. Since this absorption only partially removed the Duffy antibodies, it was subjected to a second absorption. This time the untreated Fy(a+) cells completely removed the antibodies (Table III), whereas the titers of serums absorbed with trypsinated Fy(a+), trypsinated Fy(a–), and untreated Fy(a–) cells remained unchanged, namely 16 units. Only untreated Fy(a+) cells had

TABLE III. TITRATION BY INDIRECT ANTI-HUMAN GLOBULIN METHOD OF ANTI-Fy$^a$ SERUM AFTER ABSORPTION

| | CELLS USED FOR ABSORPTION | DILUTION OF SERUM (VS. UNTREATED FY[a+] CELLS) | | | | | | | VS. FY(a–) CELLS (CONTROL) | TITER |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 4 | 8 | 16 | 32 | 64 | | |
| Prior to absorption | None | ++± | ++± | ++ | ++ | +± | + | – | – | 32 |
| First absorption | Untreated Fy(a+) | ++ | ++ | +± | – | – | – | – | – | 4 |
| | Trypsinated Fy(a+) | ++± | ++± | ++ | ++ | + | – | – | – | 16 |
| | Untreated Fy(a–) | ++± | ++± | ++± | ++ | +± | – | – | – | 16 |
| | Trypsinated Fy(a–) | ++± | ++± | ++ | ++ | + | – | – | – | 16 |
| Second absorption | Untreated Fy(a+) | – | – | – | – | – | – | – | – | 0 |
| | Trypsinated Fy(a+) | ++± | ++ | ++ | ++ | + | – | – | – | 16 |
| | Untreated Fy(a–) | ++± | ++± | ++± | ++ | +± | – | – | – | 16 |
| | Trypsinated Fy(a–) | ++± | ++± | ++ | ++ | + | – | – | – | 16 |

The reactions are rated by the number of plus signs, +++ representing the maximum of one large clump.

TABLE IV. ANTI-HUMAN GLOBULIN TEST ON CELLS USED FOR ABSORBING ANTI-Fy$^a$ SERUM

| CELLS USED FOR ABSORPTION | ANTI-HUMAN GLOBULIN TEST | |
|---|---|---|
| | CELLS OF 1ST ABSORPTION | CELLS OF 2ND ABSORPTION |
| Untreated Fy(a+) | ++ | + |
| Trypsinated Fy(a+) | – | – |
| Untreated Fy(a–) | – | – |
| Trypsinated Fy(a–) | – | – |

the ability to unite with its specific antibody. Treatment of Fy(a+) cells with trypsin so affects the cell wall that this property is lost. This was further confirmed by the results of an antiglobulin test done on the eight sets of cell sediment resulting from the two absorption experiments (Table IV); the untreated Fy(a+) cells used for absorptions were clumped, whereas the trypsinated Fy(a+) cells as well as the Fy(a–) controls, both untreated and trypsinated, showed no clumping whatsoever.

Type $Rh_1$ Fy(a+) cells were next trypsinated. A 2 per cent saline suspension of these cells was mixed with an equal volume of anti-Fy$^a$ serum and placed in a water bath at 37° C. for one hour. An antiglobulin test done on a portion of these cells was negative for clumping. The balance of the cells was washed with normal saline and reconstituted as a 2 per cent saline suspension. To 1 drop of this was added 1 drop of univalent anti-$Rh_0$ serum. After an additional hour in the water bath at 37° C., marked clumping resulted. This experiment shows that the effect of trypsin on the Duffy factor is independent of its effect on the $Rh_0$ factor.

## SUMMARY

The Duffy antibody cannot be detected by saline agglutination, albumin-plasma, or trypsinated cell methods. If untreated Fy(a+) cells are used for the test, the indirect antiglobulin test is the only known method to detect the Duffy antibody. If Fy(a+) cells, treated either with trypsin, papain, or ficin are used, they not only fail to be clumped but they also can no longer be coated with their specific antibody as evidenced by a negative indirect antiglobulin test. Trypsin-treated Fy(a+) cells and untreated Fy(a–) cells appear to react in the presence of anti-Fy$^a$ in like fashion.

Absorption experiments show that untreated Fy(a+) cells can completely absorb Fy$^a$ antibodies from immune serum, whereas the same cells treated with trypsin have completely lost this ability. Antiglobulin tests carried out on the untreated cells used for the absorption experiment are strongly clumped, whereas trypsin-treated cells used in the same manner show no clumping whatsoever.

Treatment of Duffy positive cells with enzyme interferes with their expected reaction with specific antibody. This is not mechanical in nature because after repeated washing of Fy(a+) cells with normal saline, the intensity of the indirect antiglobulin test using anti-Fy$^a$ remains unchanged. Evidence favors the conclusion that enzyme acts directly on this hemoagglutinogen and renders it unable to react with its specific antibody and that this reaction is quite inde-

pendent of its action on the $Rh_0$ factor. This is in direct contrast to the results obtained with trypsinated $Rh_0$-positive cells, the agglutinability of which, by sera containing $Rh_0$ univalent antibodies is increased.

Although the trypsin-treated cell method is of great value and the trypsinated cell indirect antiglobulin method detects antibodies in such low titer that all other methods fail, yet these methods must not be relied upon to detect the Duffy antibody. If enzyme-treated cells are used for crossmatch test preliminary to transfusion or for a screening test to diagnose sensitization, failure to detect anti-$Fy^a$ will result unless in addition the indirect antiglobulin test using untreated cells is carried out.

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# RENAL EXCRETION OF THIOCYANATE

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ALTHOUGH the value of thiocyanate therapy in the treatment of essential hypertension has been seriously questioned by many workers,[1-3] it is frequently employed because of the lack of any really adequate form of treatment. The regulation of dosage on the basis of plasma thiocyanate levels, introduced by Barker in 1936,[4] added considerably to the safety of thiocyanate therapy. However, toxic manifestations are still frequently encountered, even with blood levels in the supposedly "safe" range.[3, 5, 6, 9] In addition, the amount of drug required to maintain a "therapeutic" blood level of 8 to 14 mg. per cent varies widely in different patients, and in the same patient at different times; thus, rapid and unexpected changes in the blood level can occur.[1-5, 7] It has also been noted that toxic signs and symptoms frequently persist for several days or weeks after discontinuation of thiocyanate administration. One case has been reported in which four months were required for complete elimination of thiocyanate from the blood.[4] Most of the deaths reported from this agent have occurred at least ten days after the last dose.[8-11] The above observations suggest that elimination of the thiocyanate ion from the body may be an important factor in regulating the blood level, and that excretion of the drug may be a relatively slow process.

The thiocyanate ion is distributed in the body in essentially the same manner as chloride,[12-15] i.e., largely in extracellular fluid where it should be readily available for renal excretion. However, little is known of the manner in which the kidney handles thiocyanate. The excretion of bromide, which is also distributed in a manner similar to chloride, is dependent upon the rate of total halide excretion.[16] Although the kidney is capable of a partial discrimination between bromide and chloride, administration of large amounts of chloride produces a marked increase in bromide excretion.

Because of the similarity between the distribution of thiocyanate, bromide, and chloride, it might be expected that thiocyanate excretion would also be dependent upon total halide excretion. To test this possibility, the rate of elimination of the thiocyanate ion was determined in dogs at various levels of chloride excretion.

## METHODS

Diuresis was established in dogs under sodium pentobarbital anesthesia by the oral administration of 40 ml. per kilogram per hour of water, followed after various periods of time by the intravenous infusion of 2 to 5 ml. per minute of 1.8 per cent NaCl solution. This procedure allowed a wide variation in the rate of chloride excretion in each animal.

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A single dose of sodium thiocyanate calculated to give plasma levels of about 10 mg. per 100 ml. was injected intravenously. The actual maximum plasma values obtained varied from 7.0 to 12.2 mg. per cent. Individual experiments lasted six to twelve hours.

At various intervals urine was collected by catheter over a ten-minute period. Venous blood was drawn for plasma thiocyanate determination at the midpoint of each period of urine collection. The bladder was washed with measured amounts of distilled water before and after each collection, and the second wash was added to the urine collected. Plasma thiocyanate levels were determined in a photoelectric colorimeter by the method of Powell,[17] and the same method was adapted for use on urine by treating 2.0 ml. of urine with 2.0 ml. of acidified $Fe(NO_3)_3$ and diluting to 10 milliliters. Chesley[18] showed that some urinary pigments may interfere with thiocyanate determinations in urine. However, it was found that the amount of urinary pigment present under the

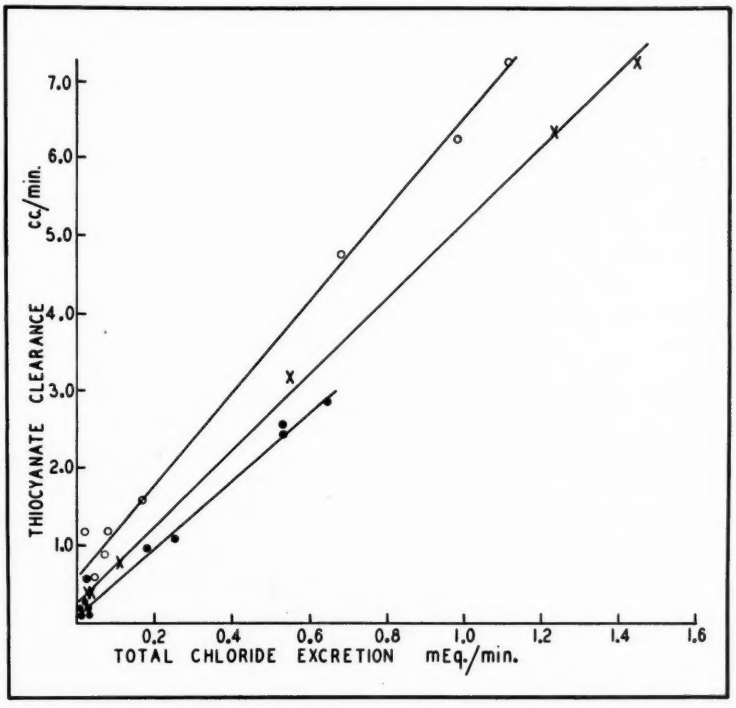


Fig. 1.—Representative experiments illustrating the dependence of thiocyanate clearance upon total chloride excretion. Selected to show maximum differences in the slopes of the lines.
×——× Dog no. 3, 9.9 kilograms.
o——o Dog no. 5, 10.2 kilograms.
●——● Dog no. 7, 9.8 kilograms.

conditions of our experiments was small in proportion to the amount of thiocyanate excreted, although this pigment may have contributed to the somewhat high thiocyanate values occasionally encountered at very low clearance rates. Urine chlorides were determined by the method of Volhard and Arnold.[19] From the above determinations, thiocyanate clearance, total milliequivalents of chloride excreted, and rate of urine flow were calculated.

## RESULTS

A total of sixty thiocyanate clearances were determined on eight dogs. Clearance values varied from 0.1 to 7.3 ml. per minute. Chloride excretion

TABLE I

| DOG (WEIGHT) | TIME AFTER $SCN^-$ INJECTION (MIN.) | PLASMA $SCN^-$ (MG. %) | URINE $Cl^-$ (MEQ./MIN.) | $SCN^-$ CLEARANCE (ML./MIN.) | URINE FLOW (ML./MIN.) |
|---|---|---|---|---|---|
| 2 | 100 | 7.0 | 0.003 | 0.2 | 2.0 |
| (7.7 kg.) | 143 | 6.8 | 0.01 | 0.2 | 3.2 |
| | 178 | 6.8 | 0.04 | 0.2 | 3.7 |
| | 195* | — | — | — | — |
| | 228 | 5.7 | 0.11 | 1.1 | 5.9 |
| | 267 | 5.4 | 0.38 | 2.8 | 6.3 |
| | 308 | 5.0 | 0.91 | 6.2 | 8.9 |
| 5 | 60 | 9.8 | 0.17 | 1.6 | 5.3 |
| (10.2 kg.) | 120 | 9.4 | 0.03 | 0.6 | 4.9 |
| | 175 | 9.3 | 0.03 | 0.6 | 3.6 |
| | 240 | 9.0 | 0.02 | 1.2 | 3.8 |
| | 255* | — | — | — | — |
| | 300 | 9.6 | 0.08 | 1.2 | 3 8 |
| | 355 | 7.9 | 0.68 | 4.8 | 3.3 |
| | 415 | 7.4 | 1.11 | 7.3 | 4.7 |
| | 470 | 7.0 | 0.98 | 6.2 | 4.1 |
| 7 | 65 | 9.6 | 0.03 | 0.2 | 4.7 |
| (9.8 kg.) | 125 | 9.2 | 0.02 | 0.3 | 3.7 |
| | 185 | 8.6 | 0.01 | 0.2 | 4.9 |
| | 245 | 8.4 | 0.01 | 0.1 | 2.9 |
| | 305 | 8.2 | 0.03 | 0.6 | 3.0 |
| | 365 | 7.8 | 0.02 | 0.1 | 4.6 |
| | 375* | — | — | — | — |
| | 425 | 7.5 | 0.07 | 0.9 | 4.6 |
| | 485 | 6.8 | 0.18 | 1.0 | 2.7 |
| | 545 | 6.6 | 0.25 | 1.1 | 1.2 |
| | 605 | 6.2 | 0.53 | 2.5 | 2.8 |
| | 665 | 6.1 | 0.64 | 2.9 | 3.0 |
| | 725 | 5.6 | 0.53 | 2.6 | 2.4 |

*Beginning of 1.8 per cent NaCl infusion.

varied from 0.003 to 1.42 meq. per minute and urine output from 0.8 to 9.0 ml. per minute.

Plots of thiocyanate clearance against total chloride excretion demonstrated a straight-line relationship for each animal, although the slopes varied slightly (Fig. 1). Thiocyanate clearance did not appear to be related to rate of urine flow, as may be seen from the representative data presented in Table I. In both the table and figure, plasma thiocyanate levels have been recorded in milligrams per cent rather than in milliequivalents to facilitate comparison with data in the current clinical literature.

Coefficients of correlation for the relationship of thiocyanate clearance to chloride excretion, calculated for each animal according to the method for ungrouped data, fell between +0.91 and +0.99. Those for the relationship of thiocyanate clearance to urine volume varied from –0.03 to +0.92. When these individual coefficients were combined by the "squared r" method, the partial coefficient of correlation for thiocyanate clearance and chloride excretion was found to be +0.92, and that for thiocyanate clearance and urine output +0.33. These values confirm the impression that thiocyanate excretion is closely related to chloride excretion, and that it is minimally, if at all, dependent upon the rate of urine flow.

In all experiments the plasma thiocyanate levels dropped more rapidly as the thiocyanate clearance values increased. However, these data are much less reliable than the data for thiocyanate clearance because marked fluctuations

in total body water and in extracellular fluid volume occurred with the shift from water to salt diuresis. The early stages of the 1.8 per cent sodium chloride infusion were always marked by a considerable decrease in the plasma thiocyanate levels (see Table I), probably largely due to dilution resulting from the increased extracellular fluid volume.

## DISCUSSION

The above results indicate a dependence of thiocyanate clearance from the blood stream upon chloride, or probably more precisely total halogenoid, excretion in the dog. Obviously the possibility of some correlation of thiocyanate excretion with one or more of the wide variety of other substances excreted by the kidney has not yet been directly excluded. However, the almost perfect correlation between thiocyanate clearance and chloride excretion over a wide range strongly suggests that other factors are of relatively minor importance. The slight correlation between thiocyanate clearance and rate of urine flow is probably incidental to the fact that high levels of chloride excretion were usually associated with a considerable diuresis.

On the basis of the present observations it appears probable that the wide variations in the required maintenance dosage of thiocyanate reported in the clinical literature are dependent to a considerable extent upon variations in the chloride intake of the patients concerned. Variations in renal function may be secondarily involved, but these are probably significant only when renal function is impaired sufficiently to allow an accumulation of both chloride and thiocyanate. Wald, Lindberg, and Barker[7] found little correlation between thiocyanate and urea clearances except when kidney function was markedly depressed. However, their figures do show very low thiocyanate clearances in patients with hypertension or nephritis, even when urea clearances were good. Our results suggest that this observation might be explained on the basis that these individuals were probably maintained on a limited salt intake.

Inasfar as the present results may be transferred to man, they suggest certain modifications in the control of thiocyanate therapy. It would appear that more consistent thiocyanate blood levels could be obtained by taking into consideration the nature of the patient's diet and its total halogen content in determining the dosage of thiocyanate to be administered. In addition, thiocyanate levels should be followed with particular care at times when the patient's diet is undergoing any considerable fluctuation.

When thiocyanate intoxication develops, administration of large amounts of chloride would appear to be the most efficacious method of obtaining a rapid excretion of the offending ion. In the few reported cases of thiocyanate toxicity where efforts to hasten excretion are recorded, large volumes of parenteral dextrose (presumably in water) were employed.[10] The results were disappointing, and the data presented above indicate that this is one of the least desirable forms of therapy, because thiocyanate clearance is very low in the presence of a water diuresis. Administration of chloride-containing solutions is a more rational form of therapy. In patients whose cardiovascular status contraindicates the administration of large amounts of sodium, other chloride salts might be employed.

## SUMMARY AND CONCLUSIONS

Thiocyanate clearance and its relation to chloride excretion were determined in dogs. Plasma thiocyanate levels comparable to those encountered clinically and wide variations in chloride excretion were studied.

An almost perfect correlation between total chloride excretion and thiocyanate clearance was obtained, but the clearance showed little relation to the rate of urine flow. The partial correlation between thiocyanate clearance and total chloride excretion was found to be +0.92, whereas that between rate of urine flow and thiocyanate clearance was only +0.33.

It is concluded that the rate of thiocyanate elimination by the kidney is dependent upon the amount of chloride excreted. It is suggested that the halogen content of the diet be considered in determining the dose of thiocyanate to be administered, and that chlorides be administered in the therapy of thiocyanate intoxication.

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# STUDIES OF CATION EXCHANGE RESINS; THEIR OPTIMAL POTASSIUM CONTENT FOR CLINICAL USE

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IN 1946 Dock[1] suggested that cation exchange resins might be used to withdraw or withhold sodium from the body in edema. Since that time there has been considerable interest in the examination of his suggestion, both in the laboratory and in the clinic. It has been established in the laboratory[2-5] that resins materially reduce the extent of absorption of sodium and potassium, but have much less effect on calcium absorption. The amount of sodium adsorbed by resins in vivo depends on their capacities and on the mineral content of the diet; usually 0.8 to 1.2 meq. of sodium and 0.4 to 0.8 meq. of potassium are bound per gram of resin. Rats have consumed diets containing 10 per cent resin, in the H-, Na-, or $NH_4$- form, over long periods of time without developing detectable symptoms of nutritional deficiency.[4, 5]

The laboratory findings have been well substantiated in the clinic; each gram of resin ingested by a human subject prevents the absorption of from 1 to 1.5 meq. of both sodium and potassium (usually slightly more of the former).[6, 7] The dose of resin most generally used is about 50 Gm. per day. When taken in conjunction with a diet of moderate sodium content, this amount of resin usually leads to the prompt relief of edema, even in cases in which mercurial diuretics are ineffective.[8, 9] The principal complications which result from resin therapy are acidosis and potassium deficiency,[6-12] although some instances of sodium and calcium deficiency have been recorded.[9, 12] The acidosis is of the compensated metabolic type,[6] and is not considered undesirable per se, since the administration of $NH_4Cl$ has long been considered an acceptable supporting therapeutic measure in cardiac edema. Potassium deficiency apparently occurs quite frequently, and general weakness results when this element is withdrawn from muscle and replaced by sodium.[7] This is particularly likely to occur on low sodium diets,[9] since the cation exchange resins generally have greater affinities for potassium than they do for sodium,[13] and, under these conditions, large fecal losses of potassium may occur. This situation may be met by frequent potassium supplementation,[6] such as by potassium-containing salt substitutes. The suggestion has also been made (see [4], text footnote 9) that the resins may be fed partly in the potassium cycle, thereby substituting potassium for some of the sodium they remove. In 1950 Kahn and Emerson[12] proposed that edematous patients be fed a carboxylic resin half in the potassium cycle and half in the ammonium cycle. They state that this combination will make it possible to "prevent excessive deprivation of potassium without, at the same time, interfering with the removal of sodium."

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In the light of our experience and in the absence of specific supporting evidence, this statement seemed open to some question. It should be recalled that a carboxylic resin of which 50 per cent is in the K-form contains 14 per cent potassium. To feed 50 Gm. of such a mixture would increase the daily potassium intake to 9 Gm., as against a normal intake of 2 Gm. In view of the naturally greater affinity of resins for potassium, it is difficult to see how such a resin regime could fail to be of decreased efficiency in sodium removal. Dock and Frank[7] have indicated their belief that the principle of administering K-form resins is a good one, but that some loss of efficiency must be expected. We[4] found in rats that, on a diet of adequate sodium and potassium content, feeding a carboxylic resin 13 per cent in the K-form (87 per cent in the H-form) appeared to reduce by 45 per cent the amount of sodium bound per gram of resin, as compared to the results obtained with 100 per cent H-form resin. In a comparison of the same two resin combinations in rats receiving a very low sodium diet, the former was found to bind 28 per cent less sodium per gram. The purpose of the work reported in this paper has been to explore Kahn and Emerson's suggestion further, and to determine whether an optimal potassium content for a resin regime can be definitely established. If it can be, two of the most serious objections which have been raised to resin therapy (potassium deficiency and acidosis) would be eliminated, at least in part.

## IN VITRO EXPERIMENTS

It was felt that a considerable amount of pertinent information could be gained from some simple in vitro studies. Accordingly, buffer solutions of the following composition were prepared:

| | *Solution I* | *Solution II* |
|---|---|---|
| $KH_2PO_4$ | 18.2 Gm. | 54.6 Gm. |
| $K_2HPO_4$ | 10.0 Gm. | 30.0 Gm. |
| $NaH_2PO_4 \cdot H_2O$ | 55.5 Gm. | 18.5 Gm. |
| $Na_2HPO_4 \cdot 2H_2O$ | 30.9 Gm. | 10.3 Gm. |
| Distilled water to | 5,000 c.c. | 5,000 c.c. |

These solutions were prepared with the objective of reproducing as closely as possible the concentration of cations which is believed to exist in a digesting suspension of food in the small intestine; i.e., about 200 meq. per liter.[14] The pH of both solutions is 6.4. When analyzed in a Perkin-Elmer flame photometer, Solution I was found to contain 147 meq. sodium and 48 meq. potassium per liter; Solution II contained 49 meq. sodium and 147.5 meq. potassium per liter. In Solution I the sodium/potassium ratio is about that in the normal human dietary, while in Solution II the ratio is one which might conceivably occur on a low-sodium, high-potassium diet. Otherwise, the above solutions obviously differ in composition from the intestinal fluids in the absence of chlorides, sulfates, and calcium, but results obtained with them should, nevertheless, be valuable as a basis for comparing the actions of the various resins which are available, and for predicting their behavior in vivo.

It is evident, of course, that the electrolyte concentrations of a mass of food in the intestine would be modified considerably by admixture with the digestive fluids, in which sodium predominates by a large margin.[14] However,

in vivo experiments[3, 4, 6] have indicated that this predominance does not have much effect on the outcome of the exchange reaction. Both Dock and Frank[7] and Hegsted and associates[18] interpret this to mean that the actual exchange occurs mostly high in, and does not continue throughout the length of, the intestine. More recent work[19] has shown that this view will probably have to be modified.

*Procedure.—*

Amberlite XE-64 was converted to the K-form by shaking 50 Gm. for five hours with 1,100 c.c. of 10 per cent potassium hydroxide. The product was filtered, washed thoroughly with water, and dried at 37° C. Analysis by flame photometry showed the potassium content to be 21.3 per cent, indicating that the conversion to the K-form was 76 per cent complete.*

TABLE I. SODIUM AND POTASSIUM UPTAKE OF SOME CATION EXCHANGE RESINS IN VITRO, AT pH 6.4

| RESIN | CYCLE | GM./L. | CHANGE IN COMPOSITION OF SUPERNATANT | | | CATION UPTAKE, MEQ./GM. | POTASSIUM AFFINITY† |
|---|---|---|---|---|---|---|---|
| | | | Na* | K* | pH | | |
| *Solution I.* | | | | | | | |
| Win 3000 | $NH_4$ | 8 | -2.8 | -1.2 | 0 | 4.0 | 1.41 |
| Win 3000 | $NH_4$ | 16 | -2.2 | -1.0 | 0 | 3.2 | 1.59 |
| Amberlite XE-64‡ | $NH_4$ | 10 | -1.5 | -0.6 | +0.1 | 2.1 | 1.29 |
| Amberlite XE-64 | H | 6 | | | | | |
| Amberlite XE-64 | H | 8 | -2.3 | -0.9 | -0.4 | 3.2 | 1.23 |
| Amberlite XE-64 | H | 16 | -1.5 | -0.7 | -0.5 | 2.2 | 1.55 |
| Amberlite XE-66‡ | H | 16 | -2.3 | -1.2 | -2.4 | 3.8§ | 1.67§ |
| Amberlite IR-105G‡ | H | 16 | -2.1 | -0.8 | -1.2 | 2.9 | 1.17 |
| Ionac C-200‖ ¶ | H | 16 | -1.5 | -0.5 | -0.5 | 2.0 | 1.05 |
| Nalcite MX¶ | H | 16 | -2.2 | -0.9 | -2.2 | 3.1 | 1.35 |
| Dowex 50 | H | 16 | | | | | 2.01** |
| Win 3000 | $NH_4$ | 8 | -2.2 | -0.9 | -0.4 | 3.1 | — |
| Amberlite XE-64 | H | 8 | | | | | |
| Win 3000 | $NH_4$ | 8 | -2.0 | -0.2 | -0.3 | 3.1†† | — |
| Amberlite XE-64 | H | 6 | | | | | |
| Amberlite XE-64 | K | 2 | | | | | |
| Win 3000 | $NH_4$ | 8 | -2.0 | +0.5 | 0 | 3.3†† | — |
| Amberlite XE-64 | H | 4 | | | | | |
| Amberlite XE-64 | K | 4 | | | | | |
| *Solution II.* | | | | | | | |
| Win 3000 | $NH_4$ | 8 | -0.1 | -3.1 | 0 | 3.2 | 11.9 |
| Win 3000 | $NH_4$ | 16 | -0.3 | -2.7 | 0 | 3.0 | 3.6 |
| Amberlite XE-64 | H | 8 | 0 | -2.4 | -0.4 | 2.4 | 8 |
| Amberlite XE-64 | H | 16 | -0.1 | -1.6 | -0.5 | 1.7 | 6.0 |
| Amberlite XE-66 | H | 16 | -0.3 | -2.9 | -2.2 | 3.7§ | 2.3§ |
| Amberlite IR-105G | H | 16 | -0.5 | -2.0 | -0.9 | 2.5 | 1.4 |
| Ionac C-200¶ | H | 16 | -0.4 | -1.3 | -0.5 | 1.7 | 1.1 |
| Dowex 50 | H | 16 | | | | 3.3 | 1.3** |

* As meq. removed, per gram of resin.

†Relative to sodium affinity; i.e., $\frac{(\text{K uptake} \times \text{final Na. core.})}{\text{Na uptake} \times \text{final K core.}}$

‡Kindly supplied to us by Dr. J. C. Winters of Rohm and Haas Co., Philadelphia.

§After acid treatment this resin still contained 0.25 meq. sodium per gram; this is included in the calculations of cation uptake and potassium affinity.

‖Kindly supplied to us by Mr. L. W. Miller of American Cyanamid Co., Stamford, Conn.

¶40-mesh.

**As supplied in small pellets; acid-regenerated.

††Including potassium introduced with the resin.

*It has come to the attention of the author that a fully-saturated potassium carboxylate resin is supposed to contain 18.7 per cent potassium. The moisture content of the carboxylic resin herein used was 10 per cent, and the anhydrous potassium salt contained 23.9 per cent potassium, or 6.1 meq. per gram; free potassium hydroxide was negligible. At complete theoretical saturation the dry resin should contain 7.2 meq. potassium per gram.[16]

All subsequent combinations which included K-form resin were made up on the basis of this figure. The resin samples to be tested were shaken mechanically for three hours with Solution I or II, the proportions being either 8 or 16 Gm. resin per liter of solution. The suspensions were then centrifuged or filtered, and analyzed in the flame photometer. The results are given in Table I.

While the list does not include all of the cation exchange resins currently available, some of the most representative have been tested. Amberlite XE-64 is the only carboxylic resin represented, but it has at least as high a capacity as any resin of this type.[16] Six different sulfonic resins have been studied, one of them in the ammonium cycle. Their comparative behavior could have been rather well predicted from the manufacturer's claimed capacity,[16] except as regards their relative affinities for sodium and potassium. The capacities of all but Ionac C-200 and Amberlite IR-105G are given as between 4 and 5 meq. per gram.

In the three-hour reaction time, none of the resins removed from the solutions an amount of cations equal to its claimed capacity. The sulfonic resins in general removed the equivalent of 70 to 80 per cent of their claimed capacities, while the carboxylic resin removed the equivalent of only 20 to 30 per cent of its potential capacity. However, the data of Kunin and Barry[15] indicate that at pH 6.4 and a salt concentration of 200 meq. per liter the effective capacity of this particular resin is only 5 meq. per gram. Making allowance for this factor, its total cation uptake under the experimental conditions was about 50 per cent of the expected amount.

As regards relative sodium and potassium affinities, the resins showed considerable variations. Ionac C-200 seemed to have the greatest relative affinity for sodium followed by Amberlite IR-105 G.; it is, however, very difficult to distinguish between some of the products. Rating the various resins in the order of increasing relative potassium affinity, they seem to be about as follows: Ionac C-200, Amberlite IR-105G, Nalcite MX, Amberlite XE-66, Dowex 50, Win 3000(Katonium*), and Amberlite XE-64. Amberlite XE-66, Win 3000, and Dowex 50 have the highest available capacities, and are essentially equal in this regard.†

The experiment involving the reaction of mixed $NH_4$-, H-, and K-form resins gave some significant results. In the first place, it is evident that at pH 6.4 and 0.2 normal salt concentration, the carboxylic K-form resin simply gave up to the solution most of its potassium, and did not replace it with sodium. In other words, even though equilibrium was being approached from the direction of the salt form, the available capacity of the resin was not appreciably greater than when it was approached from the direction of the acid form. At about 16 per cent K-form, i.e., 1.15 meq. per gram resin, the potassium uptake of the mixture would be zero. The sodium uptake of the mixed carboxylic and

*Trade-mark of Winthrop-Stearns Inc., brand of cation exchange resin.

†The actual capacity of this lot of Win 3000, as determined by equilibration with 10 per cent sodium sulfate solution, distillation after the addition of sodium hydroxide, and titration of the liberated ammonia, was 4.56 meq. per gram. On this basis, the H-form resin would have a capacity of 4.9 meq. per gram, and the K-form would contain 15.8 per cent potassium, or 4.13 meq. per gram. Hegsted and associates[18] have reported the effective in vitro capacity of this resin as 3.5 meq. per gram, which is in agreement with the data presented in Table I.

sulfonic resins was only slightly decreased by the presence of potassium in the former. However, the situation was not strictly comparable to that in the intestine. The difference lies in the fact that (for example), with 25 per cent of the total resin in the K-form, the ratio of sodium to potassium present in resin and solution was decreased only from 3:1 to 2:1. As has been mentioned previously, to feed the amount of K-form resin suggested by Kahn and Emerson would change the ratio of potassium to sodium ingested from 1:3 (as meq.) to about 4:3, and the results obtained with Solution II make it perfectly clear what would happen in this situation; i.e., the resins would pick up 5 to 6 meq. of potassium to 3 meq. of sodium. This point will be touched on below in connection with the in vivo experiments.

On theoretical grounds[16] it seems indicated that sulfonic and carboxylic resins fed in the form of ammonium or alkali salts would behave quite differently in the stomach. The sulfonate resin, behaving essentially like a neutral salt, would be expected to give up only a small amount of its alkali for hydrogen ions, but would react with the gastric juice to exchange the various alkali ions for one another. The carboxylic resin, on the other hand, would be expected to react like an alkali bicarbonate, giving up its alkali for hydrogen ions, and not taking up any other alkali ions. This was demonstrated for the potassium and ammonium forms of the two resins, as follows: Solution A was simply 0.1 N HCl, and solution B had the approximate composition of human gastric juice.[17] These solutions were shaken with the various resin combinations* for two hours at room temperature, then filtered. Ammonia was determined on the filtrate by nesslerization, sodium and potassium by flame photometry, and HCl by titration with bromphenol blue indicator. The data are given in Table II.

TABLE II. REACTIONS OF CARBOXYLIC AND SULFONIC RESINS IN THE AMMONIUM AND POTASSIUM CYCLES WITH 0.1 N HCl AND AN ARTIFICIAL GASTRIC JUICE

| TYPE OF RESIN | AMOUNT ADDED, GM./LITER | | | NET CHANGE IN COMPOSITION, MEQ. PER LITER | | | | FINAL pH |
|---|---|---|---|---|---|---|---|---|
| | H-FORM | $NH_4$-FORM | K-FORM | H | $NH_4$ | Na | K | |
| | | | | 0.1 N HCl | | | | |
| Sulfonic | 0 | 1.15 | 6.85* | -17.3 | + 0.4 | 0 | +16.6 | |
| Carboxylic | 1.07 | 0 | 3.33† | -22.3 | 0 | 0 | +23.0 | |
| Carboxylic | 3.30 | 4.80‡ | 1.90† | -47.0 | +32.1 | 0 | +13.7 | |
| | | | | Artificial gastric juice§ | | | | |
| Sulfonic | 0 | 9.2 | 3.30* | -16.0 | +25.5 | -13.0 | + 4.0 | 1.3 |
| Sulfonic | 0 | 12.0 | 0 | -15.0 | +37.0 | -13.0 | - 7.3 | 1.3 |
| Carboxylic | 8.55 | 0 | 1.90† | -14.0 | 0 | 0 | +14.0 | 1.3 |
| Carboxylic | 3.30 | 4.80 | 1.90† | -48.0 | +35.8 | 0 | +13.5 | 1.7 |

*Potassium content, 4.13 meq. per gram.

†Potassium content, 7.2 meq. per gram.

‡The ammonium-form was prepared by shaking 5 Gm. H-form resin with 200 c.c. of 20 per cent $NH_4Cl$ in 30 per cent $NH_4OH$ for eight hours, letting stand overnight, then filtering, washing thoroughly with water, and drying in the air. Analysis indicated 63 per cent conversion to the ammonium-form. Of this preparation, 7.5 Gm. was used, equivalent to 4.8 Gm. ammonium-form, plus 2.7 Gm. H-form. Additional H-form resin was derived from the partially converted K-form.

§This solution contained, per liter, 750 c.c. of 0.1 N HCl, 3.5 Gm. NaCl (60 meq.), 1.12 Gm. KCl (15 meq.) and 2 Gm. 1:10,000 pepsin.

*The proportions of resin to solution are about those which one would have with a daily intake of 50 Gm. resin, and a daily output of 4 liters of gastric juice.

The results show that the carboxylic resin, as expected, does give up practically all of its alkali to 0.1 N HCl, or to artificial gastric juice. On the other hand, the sulfonic resin in the K-form gives up only 80 per cent of its potassium to 0.1 N HCl, and in the $NH_4$-form gives up 68 per cent of its ammonia to artificial gastric juice. In the latter case, however, only about half of the alkali is exchanged for hydrogen ions, and the balance is exchanged for sodium and potassium. When a mixture of the $NH_4$- and K-forms of the sulfonic resin is shaken with artificial gastric juice, the resin gives up altogether 53 per cent of the alkali. Of this, slightly more than half is exchanged for hydrogen ions, and the balance is exchanged for sodium. The data indicate that the carboxylic resin holds a small amount of its ammonia (10 to 15 per cent) and releases all of its potassium, when shaken with artificial gastric juice or 0.1 N HCl, although, on theoretical grounds, the reverse would be expected. There is enough potential alkali in the 10 Gm. of resin combination used (40 meq. $NH_3$ and 14 meq. K) to neutralize all of the acid present in 850 c.c. of gastric juice.

Some animal experiments were now set up to compare the behavior of the potassium salts of sulfonic and carboxylic resins under physiological conditions.

*In Vivo Experiments.*—Rats weighing initially about 250 Gm. were kept in metabolism cages, one pair to each cage. They received their respective diets and water ad libitum for a period of eight days, except as noted below. The first three days were used only for the purpose of adjusting the animals to the diets, and the actual metabolic experiment was of five days' duration. The animals were deprived of food for fifteen hours before the collection of excreta was begun, and for fifteen hours before the termination of the experiment; food consumption for the intervening one hundred five hours was recorded. This was done in order that the excreta collected could be more positively identified with the food and resin intake. Feces were collected on wire screens, and the urine samples were collected in paraffin-lined bottles, using thymol as a preservative. Resin constituted 10 per cent of the diet except in the control pairs, which received none. Potassium-form resin was present in the diet of eight of the pairs; four pairs received K-form sulfonic resin (Win 3000) at levels ranging from 22.5 to 90 per cent of the total resin, and four pairs received K-form carboxylic resin (Amberlite XE-64) at levels ranging from 18 to 72 per cent of the total resin. If 90 per cent of the sulfonic resin is in the K-form, the mixture provides 3.65 meq. potassium per gram of resin. If 72 per cent of the carboxylic resin is in the K-form, the mixture provides 5.2 meq. potassium per gram of resin.

The diluted urine samples were analyzed directly for sodium and potassium in the Perkin-Elmer flame photometer, using the internal standard method. Feces were thoroughly dried, ground, and mixed; aliquots were ashed in a muffle furnace at about 1,200° F. for four to five hours, then dissolved in dilute HCl, and analyzed in the flame photometer. Food and resin samples were analyzed in the same way as feces. The basal diet used was as previously described[4]; it contained 31.4 meq. sodium, 19.6 meq. potassium, and 27 meq. calcium per

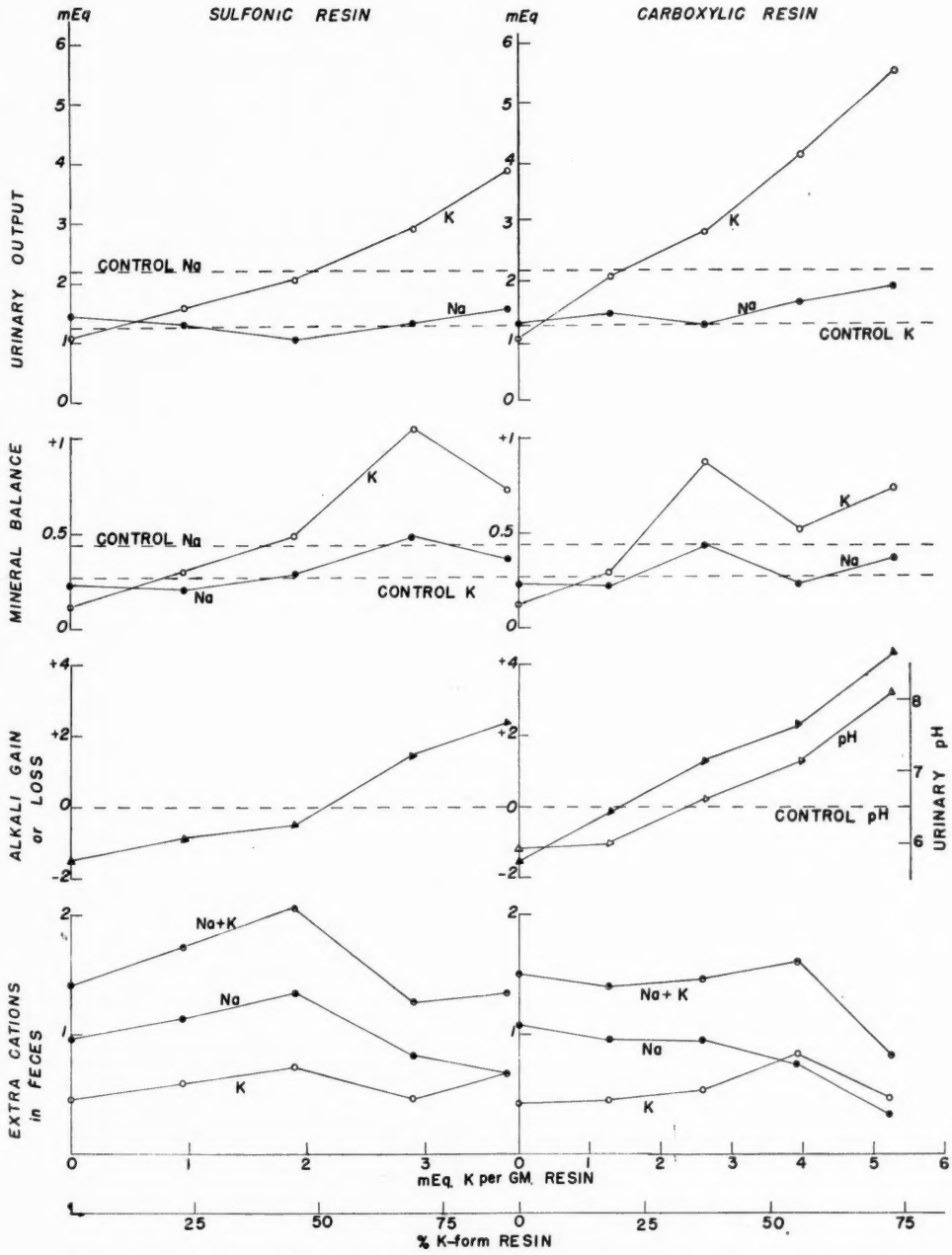


Fig. 1.—Effects of feeding two typical cation exchange resins partly in the K-form on the absorption and excretion of sodium and potassium of rats. All values plotted are in terms of meq. (i.e., 23 mg. sodium or 39.1 mg. potassium) per 9 Gm. of basal diet (1 Gm. of resin) consumed. Each point plotted represents the results on one pair of animals for one five-day metabolic period. The control values given are averages from three pairs of rats.

100 grams. From the analytical data, sodium and potassium balances were calculated for each pair of rats. The sodium and potassium bound by the resin were calculated from the extra amounts of these elements appearing in the feces as a result of resin ingestion (for details of method of calculation, see [4]), and from the amounts of resin (food) consumed. The net alkali gain or loss was taken as the difference between the terms: (extra sodium and potassium in the feces) and (potassium introduced with the resin). The effect of possible fecal calcium losses[4] is, therefore, not taken into account in arriving at these values. For purpose of convenience in evaluating the results, all of the data have been reduced to terms of meq. per 90 gram basal diet (10 Gm. resin) consumed.[3] The results are presented graphically in Fig. 1.

These preliminary experiments demonstrate that a fairly large amount of potassium can be incorporated into either type of resin without reducing its sodium-combining power too seriously. In the case of the carboxylic resin there is a steady decrease of sodium-combining power with increasing potassium content, but up to 35 per cent K-form the decrease is nominal; in the case of the sulfonic resin it would seem inadvisable to go beyond 45 per cent K-form. There are, however, other factors to be considered in arriving at an optimal potassium content. For example, the high potassium contents seem to lead to alkali retention, and this is undesirable. The optimal content would probably be considered as one which provides an adequate potassium balance, and, preferably, either a slight acidosis, or no change in acid-base balance. When all of these factors are considered, the optimal potassium content for the sulfonic resin would seem to lie between 25 and 50 per cent K-form, while that of the carboxylic resin lies between 20 and 30 per cent; in other words, between 1 and 2 meq. potassium per gram for both.

If much faith can be put in the pH of urine samples collected over such a long term, and it is assumed that the point at which the pH curve crosses the control line represents the point at which the potassium introduced with the resin just equals the fecal loss of all cations, the total in vivo capacity of this (carboxylic) resin would be established as 2.4 meq. per gram. Of this, ions other than sodium and potassium (i.e., calcium and magnesium) would make up about 1.0 meq. The sodium plus potassium capacity of the sulfonic resin averages 1.56 meq. per gram, which is considerably higher than previously reported.[4] The experiments of Hegsted and associates[18] with this resin, using low sodium diets, indicate a total capacity of 1.5 or 2.3 meq. per gram.

Since the optimal potassium content for the sulfonic resin seemed to lie between 1 and 2 meq. per gram, it was decided to study this region more intensively. Twelve pairs of rats were used, at levels ranging from 18 to 48 per cent K-form resin, and four of the pairs were at the 32 per cent level (1.3 meq. potassium per gram resin). The experiment was conducted exactly as described above except that the NaCl content of the basal diet was reduced to 0.5 per cent, giving it a composition of 14.2 meq. sodium, 19.8 meq. potassium, and 27.3 meq. calcium per 100 grams. Similar data were obtained, and are plotted in the same manner in Fig. 2.

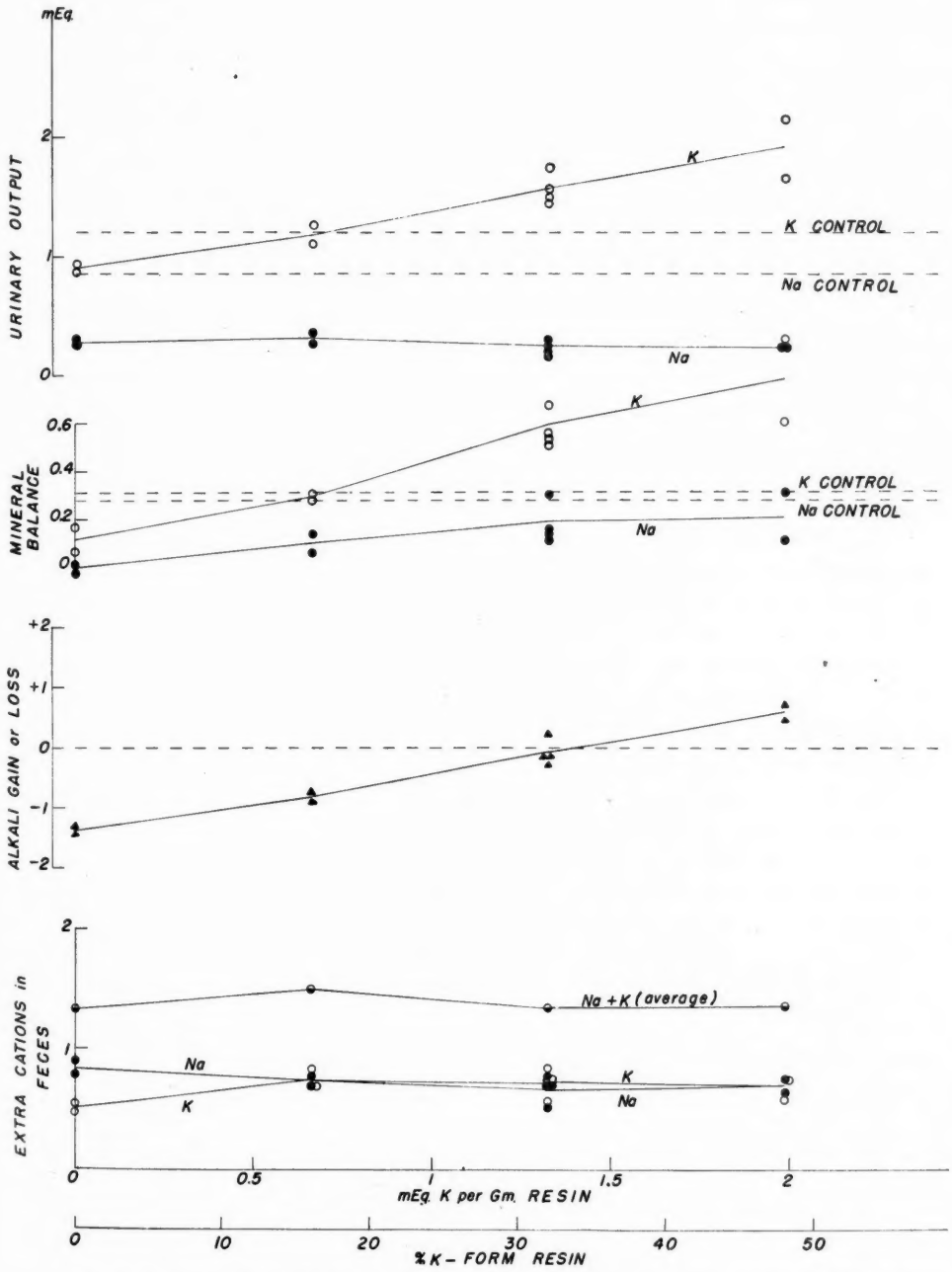


Fig. 2.—Further data on the effects of feeding a sulfonic resin partly in the K-form on the absorption and excretion of sodium and potassium of rats. All values plotted are in terms of meq. per 9 Gm. of basal diet (1 Gm. of resin) consumed. Each point represents the results on one pair of animals for one five-day metabolic period.

The results show that the sodium-combining power of the resin remains essentially constant at 0.9 meq. per gram over the entire range studied. This is a small reduction of capacity from that recorded in Fig. 1, but is to be expected from the lower sodium content of the diet; the total capacity for sodium plus potassium also diminished slightly to 1.3-1.4 meq. per gram as a consequence. The optimal point here seems to between 20 and 30 per cent K-form resin, depending upon the amount of acidosis one wishes to produce. Potassium balances are entirely adequate at 18 per cent K-form resin, or above.

## CONCLUSIONS

When tested in vitro against phosphate buffer solutions of pH 6.4, several sulfonic type resins have available capacities of 3 to 4 meq. per gram. Most of them have an affinity for potassium which exceeds by about 30 per cent their affinity for sodium. The carboxylic type of resin has an available capacity under the experimental conditions of 2 to 3 meq. per gram. At the degree of acidity of normal gastric juice the alkali salts of the sulfonic type resin are fairly stable, while the alkali salts of the carboxylic type are almost completely hydrolyzed, decreasing the gastric acidity appreciably.

If the resins are to be fed partly in the K-form, the optimal potassium content for both types seems to lie between 1 and 2 meq. per gram. In this region the loss of sodium and potassium in the feces is just about cancelled by the potassium ingested with the resin. The result is that less loss of fixed base occurs. If some degree of acidosis is preferred, the potassium content of the resin should be nearer 1 meq. per gram. The resin mixture provides an adequate potassium balance, and its sodium-combining power is only slightly impaired, if at all.

A daily intake of 12.5 Gm. K-form and 37.5 Gm. $NH_4$-form carboxylic resin (containing a total of 90 meq. of potassium) would be capable of binding about 1 Gm. sodium in the intestine,* would assure a positive potassium balance, and would result in very little net change in acid-base balance. Such a combination would neutralize about 4 liters of gastric juice of normal acidity. A daily intake of 12.5 Gm. K-form and 37.5 Gm. $NH_4$-form sulfonic resin (containing a total of 52 meq. of potassium) would be capable of binding about 1.3 Gm. sodium in the intestine,* would assure a positive potassium balance, and there would be a small net alkali loss. Such a combination would neutralize about 1,600 c.c. of gastric juice of normal acidity.

## SUMMARY

The effective in vitro capacity of a number of commercially available cation exchange resins has been determined by shaking them with 0.2 normal phosphate buffer solutions of pH 6.4. Their effect in neutralizing gastric juice, and their power to exchange alkali ions in this medium has also been studied. In vivo studies indicate that, for clinical use, the optimal potassium content of both carboxylic and sulfonic types of resins lies between 1 and 2 meq. per gram; this amount will not, however, entirely prevent acidosis.

*Based on the data in Fig. 1.

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# PROLONGED RETENTION OF BROMSULFALEIN IN PATIENTS WITH REGURGITATION JAUNDICE

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THE bromsulfalein (BSP) test has been widely employed in clinical practice, but prolonged retention in patients with jaundice has not previously been reported. The purpose of this study is to call attention to the delayed removal that follows a single intravenous injection of the dye to jaundiced patients.

## MATERIAL AND METHODS

This study includes thirty-three patients with various diseases of the hepato-biliary system. A single dose of 5 mg. of BSP per kilogram of body weight was given intravenously. All patients included in the study showed over 20 per cent BSP retention at the end of forty-five minutes. The diagnosis in twenty-seven patients was confirmed by either liver biopsy or laparotomy. In the remaining six, it was made on clinical grounds only.

In addition to the forty-five minute specimen, blood samples were taken twenty-four hours after BSP was injected. Additional observations were made whenever BSP was demonstrated in the twenty-four hour sample. The Coleman Junior spectrophotometer was used in analyzing all specimens.

TABLE I. DURATION OF BSP RETENTION IN HEPATITIS

| CASE | 45 MIN. BSP RETENTION (%) | 24-HR. BSP RETENTION (%) | DURATION OF BSP RETENTION IN DAYS | BSP RETENTION ON DAY INDICATED IN COLUMN III (%) | SERUM BILIRUBIN MG./100 ML. | DIAGNOSIS |
|---|---|---|---|---|---|---|
| 1 | 61 | 27 | Over 5 | 10 | 19.3 | Acute Hepatitis |
| 2 | 55 | 8 | 2 | 3 | 5.7 | Acute Hepatitis |
| 3 | 65 | 20 | Over 12 | 7 | 18.8 | Acute Hepatitis |
| 4 | 47 | 28 | Over 6 | 10 | 8.6 | Acute Hepatitis |
| 5 | 58 | 20 | 9 | 3 | 3.1 | Acute Hepatitis |
| 6 | 50 | 18 | 2 | 5 | 3.5 | Acute Hepatitis |
| 7 | 58 | 33 | Over 4 | 7 | 5.5 | Acute Hepatitis |
| 8 | 36 | 18 | 3 | 2 | 8.6 | Acute Hepatitis |
| 9 | 67 | 25 | Over 4 | 6 | 7.2 | Acute Hepatitis |
| 10 | 76 | 39 | Over 6 | 12 | 14.0 | Acute Hepatitis |
| 11 | 61 | 22 | Over 6 | 6 | 11.2 | Acute Hepatitis |
| 12 | 83 | 45 | Over 6 | 7 | over 25.0 | Acute Hepatitis |
| 13 | 27 | 0 | Less than 1 | 0 | 2.3 | Convalescent Hepatitis |
| 14 | 23 | 0 | Less than 1 | 0 | 1.5 | Chronic Hepatitis |

## RESULTS

The results of this study are summarized in Tables I to III. In Table I, fourteen cases with hepatitis are presented. Twelve cases with acute hepatitis had BSP retention for more than forty-eight hours, and some retained dye for longer than six days. In one case each of chronic and convalescent hepatitis, prolonged retention was not noted.

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TABLE II. DURATION OF BSP RETENTION IN EXTRAHEPATIC OBSTRUCTIVE JAUNDICE

| CASE | 45 MIN. BSP RETENTION (%) | 24-HR. BSP RETENTION (%) | DURATION OF BSP RETENTION IN DAYS | BSP RETENTION ON DAY INDICATED IN COLUMN III (%) | SERUM BILIRUBIN MG./100 ML. | DIAGNOSIS |
|---|---|---|---|---|---|---|
| 15 | 80 | 45 | Over 11 | 22 | 34.3 | Carcinoma of Gallbladder |
| 16 | 85 | 65 | Over 12 | 28 | 61.0 | Carcinoma of Gallbladder |
| 17 | 75 | 45 | Over 9 | 23 | 25.0 | Carcinoma of Common Duct |
| 18 | 70 | 40 | Over 10 | 25 | 75.0 | Carcinoma of Pancreas |
| 19 | 75 | 32 | Over 3 | 10 | 21.3 | Common duct stone |
| 20 | 66 | 35 | 29 | 4 | 10.4 | Carcinoma of Pancreas |

TABLE III. DURATION OF BSP RETENTION IN CIRRHOSIS

| CASE | 45 MIN. BSP RETENTION (%) | 24-HR. BSP RETENTION* (%) | DURATION OF BSP RETENTION IN DAYS | BSP RETENTION ON DAY INDICATED IN COLUMN III (%) | SERUM BILIRUBIN MG./100 ML. | DIAGNOSIS |
|---|---|---|---|---|---|---|
| 21 | 35 | 0 | Less than 1 | 0 | 0.9 | Laennec's Cirrhosis |
| 22 | 30 | 0 | Less than 1 | 0 | 2.7 | Laennec's Cirrhosis |
| 23 | 39 | 0 | Less than 1 | 0 | 1.8 | Laennec's Cirrhosis |
| 24 | 36 | 0 | Less than 1 | 0 | 1.5 | Laennec's Cirrhosis |
| 25 | 38 | 0 | Less than 1 | 0 | 3.3 | Laennec's Cirrhosis |
| 26 | 40 | 0 | Less than 1 | 0 | 1.5 | Laennec's Cirrhosis |
| 27 | 39 | 0 | Less than 1 | 0 | 5.9 | Laennec's Cirrhosis |
| 28 | 29 | 0 | Less than 1 | 0 | 0.8 | Laennec's Cirrhosis |
| 29 | 24 | 0 | Less than 1 | 0 | 1.7 | Laennec's Cirrhosis |
| 30 | 33 | 0 | Less than 1 | 0 | 2.2 | Laennec's Cirrhosis |
| 31 | 30 | 0 | Less than 1 | 0 | 0.5 | Laennec's Cirrhosis |
| 32 | 32 | 12 | 2 | 2 | 14.5 | Cholangiolitic Cirrhosis |
| 33 | 36 | 21 | 4 | 5 | 2.6 | Secondary Biliary Cirrhosis |

*Any value of less than 1 per cent is not significant and has been recorded as zero (0).

Six cases of extrahepatic obstructive jaundice were studied (Table II). The diagnosis in these cases was confirmed by laparotomy. The duration of BSP retention was variable, but all in this group had a significant BSP residual for prolonged periods. Case 20, for example, showed dye retention for twenty-eight days.

Of the eleven cases with Laennec's cirrhosis (Table III), however, none showed a twenty-four hour BSP retention. In this group the serum bilirubin did not exceed 6.0 mg. per cent. The case with cholangiolitic cirrhosis (32) differed from those with Laennec's cirrhosis in showing 12 per cent BSP retention at the end of twenty-four hours. One case with secondary biliary cirrhosis showed a significant retention of dye at the end of four days.

## DISCUSSION

In experiments reported by Snell, Greene, and Rowntree,[1] using dogs with ligated common bile ducts, no dye was retained in the blood stream more

than a few hours after its injection. Cantarow and Stewart[2] obtained similar results in cats. Studies done in this laboratory confirm the absence of prolonged BSP retention in dogs with obstructive jaundice.[3] The evidence presented suggests that in man, when hepatic excretion of BSP is impaired by regurgitation jaundice, extrahepatic mechanisms, such as urinary excretion, are unable to remove the dye completely.

It would seem advisable, in view of these data, to obtain a preinjection sample if the BSP test is to be repeated at frequent intervals in jaundiced patients. In addition, the sample drawn twenty-four hours after injection of dye may be of value in differentiating Laennec's cirrhosis from the other types mentioned, in the presence of minimal to moderate icterus.

## SUMMARY AND CONCLUSIONS

1. Bromsulfalein remains in the blood stream for prolonged periods following a single intravenous injection of dye to patients with regurgitation jaundice.

2. If the BSP test is to be repeated in a jaundiced patient, therefore, it would seem advisable to analyze a preinjection sample.

The author wishes to thank Dr. Victor M. Sborov for his critical review of the material presented.

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# EXPERIMENTAL PRODUCTION OF A SYNDROME RESEMBLING TOXEMIA OF PREGNANCY

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DESOXYCORTICOSTERONE acetate (DCA) in uninephrectomized rats on a high sodium diet elicits diuresis, sodium retention, and functional and morphological changes characteristic of hypertensive vascular disease.[1, 2] Renin, a specific proteolytic renal enzyme, and angiotonin, the reaction product of renin and renin-substrate, elicit diuresis and sodium loss.[3] Both sodium-retaining adrenal steroids (mineralo-corticoids or desoxycorticoids) and the renal pressor system (renin, renin-substrate, angiotonin) have been attributed roles in the pathogenesis of hypertension.[4] These facts, the stimulatory effects of desoxycorticosterone and of renin and angiotonin on water excretion, and their dissimilar actions on sodium balance lead us to study the effects of their concurrent administration.

## EXPERIMENTAL

*Renin Treatment of DCA-Hypertensive Rats.*—Sprague-Dawley female albino rats averaging 150 grams body weight were unilaterally nephrectomized and divided into two groups. Group I consisted of twenty-seven animals which received two 35 milligrams subcutaneous pellets of DCA; Group II of fourteen animals which were kept as untreated controls. The diet consisted of Purina Fox Chow and the drinking water of 1 per cent NaCl. The rats were placed in metabolism cages. Urine flow was recorded daily, and blood pressures were measured regularly by two methods previously described.[5, 6, 7] On the fortieth day the mean values of urine flow and blood pressure were respectively 65 c.c. and 171 mm. Hg in the DCA-treated group, and 20 c.c. and 115 mm. Hg in the control group. At that time treatment with a renin preparation was started in 16 DCA-treated rats and six of the controls. The purified renin sample used was prepared by Dr. A. A. Green. It contained 30 mg. of protein per cubic centimeter; 0.1 c.c. gave a 35 mm. Hg rise in the dog under nembutal anesthesia. The daily dose of 0.75 c.c. was administered at intervals in three subcutaneous injections of 0.25 cubic centimeter.

The control rats reacted to renin injections by an increase in urine output from 24 c.c. to 78 c.c. within the first twenty-four hours. The DCA-treated rats responded very differently. Four died; six survived, but were edematous and prostrate; six showed no obvious ill-effects. Urine flow decreased in the edematous survivors (from 90 c.c. to 34 c.c.), and increased in the others (from 92 to 270 c.c.). Three more rats died on or before the fifth day of continued renin injections. Diuresis increased about fourfold in the survivors. The animals which were most edematous showed nervous symptoms which varied from ties

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TABLE I. COMPARATIVE EFFECTS OF DCA

| GROUP | TREATMENT | BODY WEIGHT (GM.) | | BLOOD PRESSURE (MM. Hg) | | URINE FLOW (C.C. PER DAY) | |
|---|---|---|---|---|---|---|---|
| | | A | P | A | P | A | P |
| I | — | 170 [155-180] | 175 [160-190] | 127 [112-145] | 127 [110-140] | 15 [14-16] | 15.6 [11-22] |
| II | DCA | 164 [160-170] | 171 [161-180] | 161 [132-190] | 172 [130-200] | 56 [46-75] | 55 [35-75] |
| III | DCA and renin | 174 [165-180] | 199 [175-231] | 161 [134-180] | 187 [160-205] | 94 [60-115] | 63 [17-155] |

*Observations of body weight, arterial pressure, and urine flow before (*A*) and after (*P*) renin treatment in rats of group III, or (*P*) in groups I and II at comparable intervals. Other values are final or post-treatment. Values stated are means and ranges, the latter in brackets.

and hyperexcitability to convulsions. Attempts at indirect measurements of arterial pressure were unsuccessful because of the edema of both the tail and the paws.

At autopsy the subcutaneous tissues were edematous, and fluid had accumulated in the abdominal and pleural cavities. The volume of ascitic fluid ranged up to 15 c.c. There were diffuse and focal hemorrhages in the kidney, heart, adrenals, liver, intestine, bladder, thymus, and brain. In some, the gastrointestinal tract was filled with bloody material and the urine was dark red. These changes were superimposed on the characteristic lesions of DCA-hypertensive vascular disease. Histological changes will be reported separately. The effects of renin injection in the DCA-treated animals were so sudden and so unanticipated that no blood chemical determinations were done at this time.

In order to extend the observations, three groups of ten rats each were observed under conditions identical with those described above. Group I was the untreated control; group II consisted of DCA-treated rats, and group III of DCA-treated rats which, on the twenty-first day after pellet implantation were given three separate injections of 0.25 c.c. each of renin. All the animals were sacrificed on the next day, with the exception of two animals in group III which had died in the interval.

There were no untoward changes in animals of group I and II. In group III, four rats showed anasarca, two ascites with edema of the pancreas, stomach, and mesoappendix, and two had no obvious edema. All these animals presented hemorrhages of varying degrees, which were diffuse and sometimes involved whole organs, usually the kidney and heart.

Results of various measurements are summarized in Table I. In reviewing the tabular data it is important to recall that DCA-treated animals of group III showed varying and disparate responses to renin. The means of observations are therefore not representative of changes observed. Even ranges do not fully illustrate the condition. For example, when there was an increase in body weight after renin injection, it did not necessarily occur in the heaviest rat of the group.

Body weight and urine flow were recorded at the time of the renin injection and twenty-four hours later. Rectal temperature was measured with a thermocouple. Animals were killed by bleeding and blood taken for chemical examination. Blood urea nitrogen (B.U.N.) was determined by the method of

AND RENIN AFTER DCA PRETREATMENT*

| RECTAL TEMP. | CLOTTING TIME (SECONDS) | B.U.N. (MG. %) | HEMOGLOBIN (GM. %) | SERUM K (MEQ/L) | SERUM Na (MEQ/L) | UREA CLEARANCE (C.C. PER MIN.) |
|---|---|---|---|---|---|---|
| 97.5 | 80 | 12 | 12.9 | 4.21 | 136.4 | 0.66 |
| [97.2-98.4] | [45-115] | [10-20] | [11.1-14.2] | [3.9-4.9] | [133-140] | [0.38-1.06] |
| 97.9 | 107 | 21.2 | 12.3 | 2.16 | 149 | 0.46 |
| [97.1-98.5] | [90-120] | [12.8-28] | [11.7-12.3] | [1.8-2.4] | [146-151] | [0.32-0.66] |
| 91.4 | 156 | 128 | 12.8 | 3.8 | 153 | 0.085 |
| [87-99] | [120-180] | [30-150] | [11.9-14.5] | [1.95-5] | [149-154] | [0.04-0.15] |

Archibald,[8] K and Na by flame photometry, and blood clotting time by the capillary tube method. Urea clearance was calculated from urinary output of urea before and after renin treatment, blood urea, and urine volume.

As can be seen from Table I, changes in body weight, urine flow, and rectal temperature occurred only in group III. These showed increased body weight and decreases in urine flow and body temperature. The fall in temperature was especially severe in those animals with severe anasarca. These changes were sometimes much more profound than average values would indicate, because some animals did not show any obvious water retention and therefore no increase in weight, no oliguria, and no fall in temperature. Clotting time was almost doubled in group III in comparison with control group I; there was a small but questionable increase in group II. Hemoglobin did not change. B.U.N. was increased in group III in proportion to oliguria. As expected, serum Na was increased and K decreased by DCA treatment (group II); however, concentrations of both electrolytes increased when renin was given. These increases occurred in the oliguric rats. The deleterious effects of DCA-renin treatment on renal excretory function are illustrated by low rates of urea clearance in group III.

Since indirect measurements of arterial pressure were unsatisfactory in animals which had become grossly edematous, this function was measured in animals from each group and, in animals of group III, before and four hours after the injection of the first dose of renin (Table I). It is seen that a single dose of 0.25 c.c. of the renin preparation had a pressor effect in animals pretreated with DCA. No considerable changes of arterial pressure occurred in animals of group I and II, observed at the same times. The pressor effect of renin was not tested at this time in animals of group I. Subsequently, six rats were prepared under conditions identical with those of group I, and blood pressures measured before and four hours after renin injection. The control mean value was 126 mm. Hg (range 110-140) and, after renin the mean was 145 (range 140-155). These observations demonstrate that renin is indeed pressor when given subcutaneously; they do not suggest any hyperresponsiveness to the pressor effect of subcutaneous renin in DCA-hypertensive rats. However, since the single post-treatment observation was made at an arbitrary time of four hours after injection, the course of the blood pressure response in the two groups cannot be fully evaluated. Systematic studies by methods of continuous record-

ing are in progress. At least, during the onset of the changes induced by renin injection in DCA-treated rats, it is apparent that arterial pressure is increased beyond pre-existing levels.

*Concurrent Treatment with Renin and DCA.*—Since this hydropigenous syndrome was obtained in rats pretreated with DCA it was of interest to see whether concurrent treatment with DCA and renin would have the same effect.

The following experiment was devised. Twenty female albino rats were uninephrectomized and divided into three groups, of respectively six, six, and eight animals. Treatment was as follows: group I, DCA alone; group II renin alone, and group III, DCA plus renin. Renin, identical with the preparation used in the previous experiment, was given subcutaneously in two daily doses of 0.25 c.c. each. DCA in the form of water suspension was injected daily in a dose of 5 mg. contained in 0.5 c.c. All the animals were given 1 per cent saline to drink and were placed in metabolism cages. Treatment was started at the same time in all groups, and the experiment was terminated on the twenty-second day.

As can be seen from Fig. 1 diuresis increased in all groups: it was least in the rats receiving renin alone (group II) and greatest in those given both renin and DCA. None of the animals of group III died or presented evidence of water retention. The diuretic effects of the two substances were additive. Determinations of blood urea nitrogen gave average values of 17.9 for the DCA group, 12.8 for the renin group, and 8.9 mg. per cent for the combined treatment group. Determinations of urinary chlorides, creatinine, and urea were not informative. In Table II are summarized weights of adrenals, heart, and kidney, as well as initial and final body weight. Body weight did not change greatly. Animals of groups I and II grew normally. The weight increment was less in group III, which indicates that water was not being retained, and confirms the wasting effect of renin previously observed.[9] Heart weights and kidney weights were higher in the group receiving renin plus DCA; adrenals were larger in the group receiving renin alone.

TABLE II. EFFECT OF CONCURRENT TREATMENT WITH RENIN AND DCA*

| GROUP | TREATMENT | BODY WEIGHT (GM.) A | BODY WEIGHT (GM.) P | ADRENALS (MG.) | HEART (MG.) | KIDNEY (MG.) |
|---|---|---|---|---|---|---|
| I | DCA | 118 [108-132] | 148 [135-155] | 33 [30-36] | 586 [535-635] | 535 [523-550] |
| II | Renin | 124 [112-145] | 157 [135-180] | 43 [38-50] | 566 [555-585] | 504 [490-528] |
| III | DCA and Renin | 144 [119-150] | 153 [120-185] | 39 [31-50] | 649 [554-788] | 608 [506-1002] |

*(*A*) and (*P*) as in Table I.

Histologic examination of kidneys in the DCA treated rats (groups I and III) revealed early lesions of hypertensive vascular disease consisting of dilation of tubules, hyaline, or granular casts, areas of fibrosis enclosing partially hyalinized glomeruli, and focal arteriolar necrosis. From the presence of these morphological changes due to DCA, and the absence of the findings induced by renin in the first experimental series, it seems that the well-established DCA-hypertensive disease is a prerequisite to the production by renin of the DCA-renin sydrome described above.

*Angiotonin in DCA-Hypertensive Rats.*—Since angiotonin is the effector agent of renin action, its ability to elicit the syndrome was tested in the following experiment.

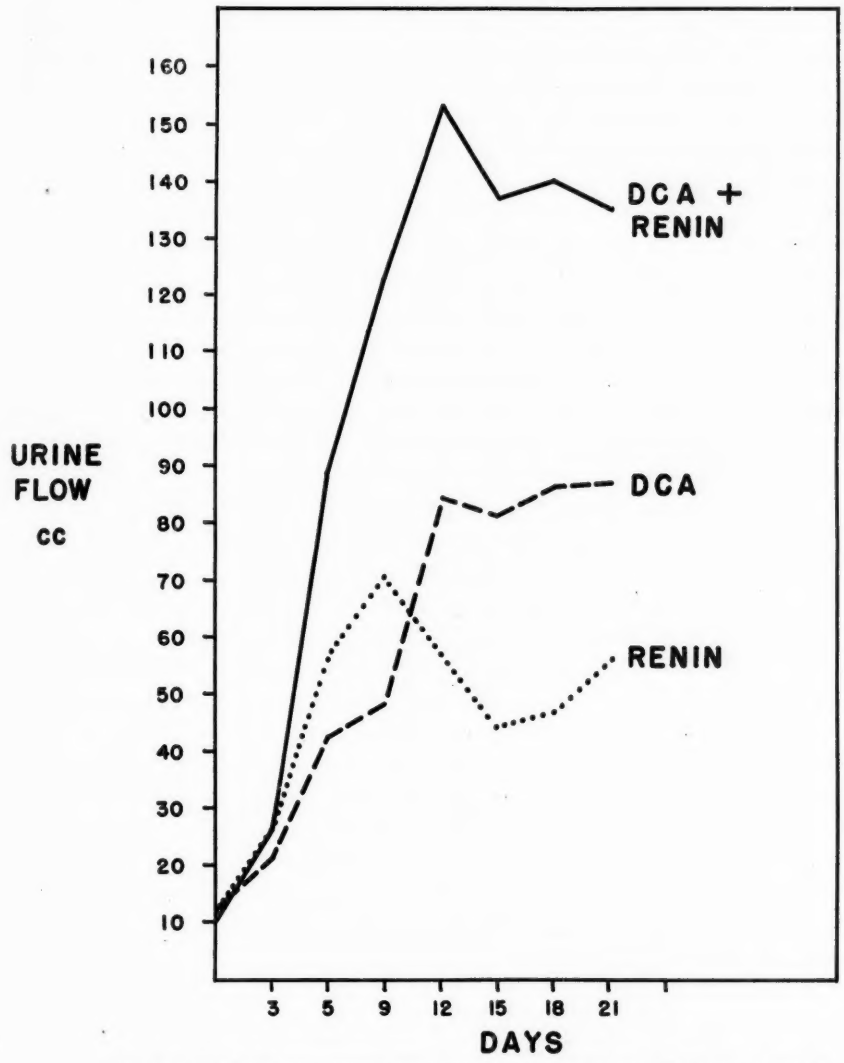


Fig. 1.—Effect of concurrent treatment with renin and DCA on diuresis.

Ten albino female rats were uninephrectomized, received two subcutaneous implants of 40 mg. each of DCA, and were kept on 1 per cent saline as drinking water. Thirty days later when the daily diuresis averaged 82 c.c. and body weight 172 Gm., five rats received two intravenous injections of 2 c.c. of angiotonin solution. This was prepared by dissolving in saline a crude powder obtained from Dr. K. Savard. The concentration was 10 mg. per cubic centimeter; in anesthetized dogs 0.2 c.c. produced a rise of 10 mm. Hg. The injections in rats were done under ether anesthesia after exposure of the jugular vein. During the next twenty-four hours the diuresis averaged 83 c.c. and the body weight 171 Gm., so that it seemed that angiotonin, in this dose, had no effect on water metabolism.

Since the effects of angiotonin given intravenously are of a very short duration, and since anesthesia could have interfered with kidney function, we decided to test angiotonin using another route of administration. All DCA-treated rats received during a period of twenty-four hours five intraperitoneal injections of 2 c.c. each of the same angiotonin preparation. It was observed that the animals became prostrate following the first injection, and remained in this condition during the day. After twenty-four and forty-eight hours the mean urine flows were 107 and 170 c.c., respectively, while the body weights were 175 and 176 grams. None of the animals presented edema, although it was apparent that angiotonin diuresis had occurred.

It remained to be shown that the same animals would have responded to renin. To this end five of the rats during the next twenty-four hours were given three subcutaneous injections of renin of 0.2 c.c. each. The mean urine flow decreased to 48 c.c.; the average body weight increased from 176 to 211 Gm. as a result of water retention. All rats were killed, including some which were moribund. Most had anasarca, hemorrhages in intestine, kidney, and brain. Microscopically we could not find any obvious proportion between the lesions resulting from DCA treatment and the intensity of the edematous reaction. In one rat which had generalized edema of the subcutaneous tissue and viscera, ascites and pleural fluid, there was no arteritis in the mesenteric and pancreatic arteries, and the kidney appeared normal; its blood urea nitrogen was only slightly elevated (28 mg. per cent). In some others, water retention and hemorrhages were not as severe, although the kidneys were enlarged, grayish, with a granular mottled appearance, and their blood urea nitrogen averaged 120 mg. per cent (range 94-130). Blood pressure was also measured in some animals before and after treatment: it was slightly higher after renin treatment (191 mm. Hg) than in the control rats (175 mm. Hg).

Thus angiotonin did not seem to have the properties of renin in eliciting edema, convulsions, oliguria, and attendant changes, although it elicited diuresis. However, the prostration induced by intraperitoneal injection of the preparation used was quite unlike the effect of subcutaneous renin. It may reflect an angiotonin contaminant which is in some way protective. Until more highly purified preparations can be obtained and a better dosage form established, the conclusion that angiotonin cannot under appropriate conditions elicit the syndrome would be premature.

*Effects of Other Substances in DCA-Hypertensive Rats.*—From the last series of experiments it seemed at least possible that some factors other than renin might be responsible for precipitating the syndrome. Those which come to mind are: (a) proteolytic enzymes, which might have some of the activities of renin or of contaminants of the renin preparation used, (b) pitressin, which is vasoconstrictor, antidiuretic, and chloruretic, and (c) thromboplastin, which has been attributed a part in the genesis of eclampsia. Each of these possibilities was examined in turn in the following experiments.

Twenty-three female albino rats received after uninephrectomy two subcutaneous implants of 40 mg. each of DCA and were kept on 1 per cent saline. On the twenty-fifth day their blood pressure averaged 150 mm. Hg (130-185) and urine flow 80 cubic centimeters.

The following day each of four rats received 6 I.U. of pituitrin in three subcutaneous injections. Except for a slight drop in diuresis from 87 to 71 c.c., the animals were not otherwise affected. Since this lack of effect could be attributed to an insufficient dose, eight days later each of six rats were given 15 I.U. of pituitrin in three subcutaneous injections. Following each injection the animals became prostrate. Within the next twenty-four hours there was a marked drop in diuresis from 84 to 45 cubic centimeters. Body weight remained stationary. Following recovery, the same animals received three injections each of 0.25 c.c. of renin. One rat was dead the following morning, while the others presented mild symptoms of water retention.

A few days after the renin injection, four rats were given thromboplastin of bovine origin intraperitoneally. The total dose of 400 N.I.H. units were subdivided into three injections. There was a slight reduction in diuresis, but no other changes.

On the following day four other rats received a proteolytic enzyme, chymotrypsin. It was administered subcutaneously in a total dose of 18 mg. subdivided into three injections. Within twenty-four hours the diuresis went down from 134 to 60 c.c., and the body weight increased from 204 to 247 Gm. All four animals showed severe and diffuse subcutaneous edema: one died during the night, and the other three were killed. At autopsy we noticed hemorrhagic edema in the subcutaneous tissue and moderate edema of the pancreas. These were the only points of similarities between rats treated with chymotrypsin and those treated with renin. The chymotrypsin-treated rats differ from the latter by the absence of water retention in abdominal and pleural cavities, of hemorrhages in the viscera, and of nervous symptoms. Their condition was similar to that which might have resulted from a severe scald.

Since the dose of chymotrypsin was obviously excessive, we repeated this experiment at a lower dose level with another proteolytic enzyme, trypsin. Three rats were injected with trypsin, and three with renin. Furthermore four unilaterally nephrectomized rats on 1 per cent saline used as controls were also injected with trypsin. The trypsin solution was prepared according to the method of Rich and Duff[10] by stirring 1 Gm. of USP trypsin powder in 20 c.c. of saline, and centrifuging to remove nonsoluble material. A daily dose of 1 c.c. of the supernatant was injected subcutaneously in each rat in two injections. The dose of renin consisted of two injections of 0.25 c.c. One of the renin-treated rats was dead the next morning, while another had severe nervous symptoms and eliminated dark red urine; at autopsy, we found the lesions described previously. On the other hand in the trypsin-treated rats, except for a slight edema at the point of injection present in the DCA as well as in the control rats, there was no effect on diuresis or body weight. At autopsy we found lesions of hypertensive vascular disease in the DCA rats, but no other lesions which could be attributed to trypsin. On histologic examination, there was necrosis of the subcutaneous tissue and hemorrhages at sites of trypsin injection, as it has been previously reported by Rich and Duff.[10]

Many of the changes incident to toxemia of pregnancy have been related to the release of thromboplastic substances from the placenta.[11] Although the experiment noted above in which thromboplastin was injected was negative, it seemed desirable to test a human placental extract rich in thromboplastin. Such an extract was obtained from Dr. E. W. Page. One cubic centimeter of this

material was injected subcutaneously four times daily in eight rats on the twentieth day of DCA treatment. Urine flow decreased the next day from 75 c.c. to 41 c.c., and in one rat from 40 c.c. to 6 c.c. In spite of the oliguria, there was no water retention; body weights before and after treatment were respectively 122 and 120 grams. Two rats were sacrificed at this time and showed neither edema nor hemorrhages. Three days later the six survivors were each given three doses of 0.25 c.c. of renin. Mean urine volume in the group decreased on the next day from 92 to 28 c.c., and body weight increased from 138 to 163 grams. One of the rats died during the night. This one and five which were sacrificed showed at autopsy all the gross lesions characteristic of the DCA-renin syndrome.

*Effects of Renin in NaCl Hypertension.*—The hypertension produced by DCA probably results primarily from the effects of this steroid on electrolyte and water metabolism. The most marked effect is sodium retention. This can be duplicated in some degree by oral administration of hypertonic NaCl solution,[12] and rats so treated develop moderate arterial hypertension.[13] It would therefore be expected that rats maintained on hypertonic NaCl should respond to renin administration much as do DCA-treated rats. In order to test this hypothesis the following experiment was devised.

Twenty-four albino female rats were uninephrectomized and divided into four groups: animals of groups I and II were kept on 1 per cent NaCl, while those of groups III and IV received hypertonic solution. Ultimately animals of groups II and IV were injected with renin. At the beginning of the experiment, the hypertonic solution consisted of 2 per cent NaCl. After a week, the concentration was reduced to 1 per cent because the animals were losing weight; then the concentration was restored gradually to 2 per cent over a period of three weeks, during which they regained some of their original weight, and urine flow reached 114 cubic centimeters. All the animals were kept on this regime for three months. At the end of this time the average blood pressures in groups I and II were 136 mm. Hg (range 125-147), and in groups III and IV, 166 mm. Hg (range 125-210). Animals of groups II and IV were then treated with renin (three injections of 0.5 c.c. during twenty-four hours). All the rats, except two of group IV which died during the night, were killed the next morning. Blood and urine were kept for analysis. Blood chlorides were determined by the method of Van Slyke and Hiller,[14] and urine proteins by the Shevky-Stafford method. Blood urea nitrogen and urea clearance were also measured as described above. The results are summarized in Table III.

The effects of renin on body weight and urine flow were not as striking as in animals pretreated with DCA, but were still present in rats on hypertonic NaCl (group IV) in which urine flow decreased and body weight increased. Edema was present in the two animals which died and in one of the survivors. On the other hand, injection of renin in animals on 1 per cent NaCl (group II) resulted in an increase in the mean urine flow and also body weight. On examination of the individual results, we noted that the response in group II was very variable, and that diuresis increased more often than decreased. However, in one such rat the body weight increased from 221 to 248 Gm. and the urine output decreased from 21 to 5 c.c.; at autopsy this rat showed anasarca, hemorrhages in kidney, pancreas, and stomach. Another rat showed a drop in urine flow from 25 c.c. to 8 c.c., but his body weight varied only slightly. The other rats

TABLE III. EFFECTS OF RENIN IN RATS PRETREATED WITH NaCl*

| GROUP | TREATMENT | BODY WEIGHT (GM.) A | BODY WEIGHT (GM.) P | URINE FLOW (C.C.) A | URINE FLOW (C.C.) P | SERUM CL (MEQ/L) | B.U.N. (MG. %) | URINE PROTEINS (GM. PER 24 HR.) | UREA CLEARANCE (C.C. PER MIN.) |
|---|---|---|---|---|---|---|---|---|---|
| I | 1% NaCl | 205 [185-232] | 194 [170-220] | 20 [15-30] | 25 [10-50] | 107 [102-112] | 13 [11.2-14.8] | 0.017 [0.004-0.078] | 0.63 [0.46-0.79] |
| II | 1% NaCl and renin | 208 [203-212] | 225 [200-270] | 20 [10-25] | 54 [5-110] | 109 [105-111] | 14.3 [8.5-20.2] | 0.120 [0.014-0.216] | 0.62 [0.50-0.75] |
| III | 2% NaCl | 145 [132-167] | 139 [122-168] | 64 [34-172] | 66 [77-140] | 130 [121-147] | 23 [12.2-37.2] | 0.028 [0.005-0.056] | 0.67 [0.52-0.78] |
| IV | 2% NaCl and renin | 146 [97-192] | 156 [100-216] | 68 [15-120] | 29 [10-50] | 127 [108-148] | 49 [22-1.04] | 0.117 [0.018-0.270] | 0.29 [0.04-0.50] |

*(A) and (P) as in Table I. Other values are post-treatment or final.

of group II showed an increase in diuresis but were not otherwise affected. In subsequent experiments, it has been found that occasionally rats on 1 per cent saline present the syndrome, but always in a mild form, and never lethally.

Blood chlorides and B.U.N. values were higher in groups III and IV than in groups I and II. Injection of renin resulted in higher values in B.U.N. in group IV than in group II, indicating a milder reaction in the latter group than in the former. As expected, proteinuria was increased by renin injection; no difference was found, however, between groups II and IV. Urea clearance was depressed in group IV but not in group II.

## DISCUSSION

The data demonstrate that renin-treated DCA-hypertensive rats commonly exhibit a syndrome consisting of hypertension, edema, oliguria, and convulsions, and which is frequently fatal. The syndrome is apparently not attributable to direct action of the steroid, since it is not elicited in rats in which renin and DCA injections are initiated concurrently. Rather, it reflects some remote effect of DCA on electrolyte metabolism, since it is elicitable—although in milder form—in rats brought into a state of sodium retention by long periods on hypertonic solutions of NaCl. Sodium retention induced by pretreatment with DCA and isotonic NaCl results in hypertension and arterial disease[1]; the sodium retention induced by prolonged administration of NaCl is also associated with hypertension, although it is less severe and arterial disease rarely a sequel.[13] Further, the onset and severity of the syndrome in renin-treated DCA-hypertensive rats cannot be correlated with the severity of the arterial disease found at autopsy, and occurs sometimes in severe form in animals in which such lesions are minimal. Thus arterial disease (nephrosclerosis and other hypertensive vascular changes) does not seem to be the anatomic substrate on which renin acts to set off the syndrome. The substrate is either one of prolonged sodium retention or of arterial hypertension and, since we are as yet unable to determine which of these factors is essential to the process, we choose temporarily to denominate the renin-susceptible state as one of the "DCA-disease," having regard to the fact that prolonged administration of hypertonic NaCl closely mimics and probably qualitatively substitutes for the action of DCA and isotonic NaCl.

The susceptible state having thus been defined as one of "DCA-disease," the question arises as to the nature and mechanism of action of the precipitating factor. Is it in fact renin? Unfortunately, chemically pure renin preparations do not exist as yet, so that the conclusions drawn from the effects of preparations containing renin must be considered provisional.

In general, it is assumed that a property of a renin preparation which can be simulated by injection of angiotonin must be attributed to renin as such. Under the conditions of our experiments with angiotonin we were unable to elicit the syndrome in rats in which renin was subsequently shown to be effective. This is prima facie evidence against renin as the factor which acts in DCA-disease to elicit the syndrome. However, as noted above, intraperitoneal injec-

tions of crude angiotonin resulted in a state of prostration unlike that produced by subcutaneous renin. Further, the action of angiotonin is in any case briefly sustained. Thus, the angiotonin preparation may have contained some contaminant which somehow protected the animal from the effect of angiotonin as such or the action may be so brief as not to duplicate that of renin. Consequently, until it is shown that the prolonged action of purified angiotonin does not have the effect of renin in DCA-disease, the possibility remains that it may duplicate the action of renin in this regard as it does in many others.

The factor responsible might not be renin, but some renin contaminants, such as proteolytic enzymes of similar properties. Two such were tested (chymotrypsin and trypsin) and found not to reproduce the action of renin. These do not exhaust the possibilities, and other catheptic enzymes must be tested or pure renin obtained before this possibility is excluded. Nevertheless, the inactivity of trypsin and chymotrypsin somewhat increases the possibility that the action is one of renin as such.

That it is probably not a sequel to nonspecific vasoconstriction is indicated by the fact that pitressin in large, and in some experiments excessive doses, did not have the action of renin.

Thus the possibility remains that the action of renin preparations in producing this syndrome in the presence of DCA-disease is a hitherto undescribed property of renin as such. It differs widely from the action of renin in normal rats, which show only polyuria, proteinuria, and weight loss.

Further suggestive evidence that it is renin which elicits the syndrome are the hemorrhagic lesions found at autopsy. Similar lesions have been produced in renal hypertensive dogs by injection of renin preparations.[15] Winternitz and associates[16] also demonstrated hemorrhagic lesions as the result of injecting renal extracts into nephrectomized dogs. From their extracts they believed that they had separated out pressor (renin), depressor, necrotizing, and hemorrhagic factors. The existence of these distinct discrete factors has not been confirmed, and our presumption, like that of Leiter and Eichelberger,[15] is that renin has the property of causing hemorrhages in animals appropriately sensitized by some form of renal damage.

We were impressed by the concurrence in the syndrome of edema, oliguria, proteinuria, hypertension, and renal excretory failure with convulsive seizures. These, in the last trimester, are the clinical characteristics of eclamptogenic toxemia of pregnancy in human beings. Hematuria and hemoglobinuria, such as were observed in some of the rats, are uncommon in toxemia; they have been noted in some cases, notably those in which toxemia is associated with abruptio placentae and in some with renal cortical necrosis.[17] The possibility is advanced that the syndrome elicited in DCA-disease by injection of renin is an incomplete experimental analogue of late pregnancy toxemia.

Its incompleteness lies (1) in that it is produced in the absence of pregnancy, and therefore of placental lesions, and (2) in that the hepatic lesions of toxemia were not found in this syndrome. These latter, however, have been related to a release of placental thromboplastin, and have been found to occur

in rabbits subjected to placental damage.[18] They would not, therefore, be expected in a syndrome elicited in nonpregnant animals. Experiments with pregnant rats and rabbits are now in progress.

The requisites of the syndrome we described are prolonged sodium retention and briefly sustained injection of renin. A variety of indications support the view that toxemia of pregnancy is associated with the concurrence of a sodium-retaining state, hypertension, and renal damage which might lead to renin release.

The sodium retention present especially in the last trimester of normal pregnancy is variously explained. The evidence at hand indicates that it is due to increased renal tubular sodium retention[19] and that this in turn is due to the presence in the body of greatly increased amounts of steroid hormones which cause the renal tubules to increase their reabsorption of this electrolyte. Two types of hormones may be responsible. The one is progesterone. It is present in late pregnancy in increased amounts. It has sodium retaining and other properties which qualitatively, if not quantitatively, are similar to those of DCA.[20, 21, 22] It has also the property of preventing the onset of labor so that in rats it causes death of the mature fetus and placental degeneration and necrosis.[23] To it has been attributed production of experimental toxemia of pregnancy in pregnant rats,[24] in that it caused albuminuria, azotemia, edema, and hypertension, as well as liver and kidney lesions. The other type of hormones are of adrenal cortical origin. In late pregnancy there is a tendency to excrete increased amounts of urinary corticoids, as determined by biological and chemical methods.[24-27] This is interpreted as indicative of adrenal hyperfunction which implies the presence of increased amounts of sodium-retaining hormonal products of the adrenal cortex.

Quite possibly both types of hormones contribute to pregnancy sodium retention. However, the current interest in the adrenals has led authors[28-32] in various countries to theoretical and loosely woven discussions which unite in suggesting that adrenal hyperfunction and the phenomena of the General Adaptation Syndrome[33] are basic factors in late pregnancy toxemia.

Our studies with DCA and renin can be viewed as confirmation of this latter interpretation. This they may be, since the concepts involved are very inclusive. However, all that can be fairly adduced from our experiments is that the concurrence of prolonged sodium retention and of briefly sustained renin injections results in a syndrome which has some of the aspects of eclamptogenic toxemia of pregnancy.

## SUMMARY

Rats with established DCA-hypertensive disease injected with a renin-containing renal extract manifest during the next day or two a syndrome of anasarca, convulsions, and oliguria or anuria. It often terminates fatally. In contrast, concurrent treatment with renin and DCA from the onset (i.e., in the absence of established DCA hypertension) elicited only the additive effect of renin on the DCA diuresis and some weight loss. The syndrome can be obtained, although in less severe form, in rats brought into mild hypertension by prolonged administration of hypertonic or, in some instances, of isotonic NaCl.

Thromboplastin of bovine or human placental origin, trypsin, chymotrypsin, or pitressin did not elicit the syndrome in DCA-hypertensive rats.

The syndrome and the associated renal anatomic changes are reminiscent of eclampsia. The hepatic changes of eclampsia are lacking, but these have been attributed to, and reproduced by placental damage, which was not a factor in these studies. The fact that eclampsia is associated with and, in some degree, determined by the concurrence of sodium retention, hypertension, and renal disease accords with the suggestion that renin treatment of DCA-hypertensive rats gives new insight into the mechanism of eclamptogenic toxemia as also into the eclamptic state which occurs in severe acute diffuse glomerulonephritis.

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# THE EFFECT OF BENEMID [P-(DI-N-PROPYLSULFAMYL)-BENZOIC ACID] ON BLOOD LEVELS OF PAS

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SINCE PAS (para-aminosalicylic acid) has become recognized as an important weapon in the treatment of tuberculosis, the report by Boger and Pitts,[4] that Benemid [*p*-(di-n-propylsulfamyl)-benzoic acid] was much more effective than carinamide in elevating plasma levels of PAS, was of unusual interest to us and led to the experiments with Benemid* described below. An attempt was made to determine: (1) the magnitude of enhancement by Benemid of blood PAS levels on our usual (10 to 12 Gm.) and reduced (6 Gm.) dosages of PAS; (2) the effect of Benemid on the ratio of acetylated to free blood aryl amine as determined by the method of Way; (3) the clinical toxicity of Benemid; (4) the relative merit of Benemid therapy, as opposed to simple changes in form and frequency of PAS administration alone, in maintaining significant blood PAS levels.

## METHOD AND PROCEDURES

Venous blood samples, drawn at specified intervals and oxalated, were kept frozen until their PAS content, both free and total, was determined by the method of Way and co-workers,[17] which measures free aryl amine and therefore is not specific for PAS. A standard curve was prepared using solutions containing from 0.1 to 10 mg. per cent of PAS. Since we were interested primarily in concentrations of 0.1 mg. per cent and higher, no special dilutions or modifications of method were made to assure greater accuracy in determination of lower concentrations; all concentrations below 0.1 mg. per cent are reported simply as "trace." Standard solutions were run with each group of blood samples. Figures for blood levels given here, unless stated otherwise, represent the concentrations of the free aryl amine, which is more tuberculostatic than any of its derivatives tested.[12, 18] Whole blood was used in all determinations; in addition, PAS content of both whole blood and plasma was determined on several samples for the purpose of comparison.

PAS blood levels were determined while patients were receiving PAS only, and then after the addition of Benemid, on several different dosage schedules. All patients had been taking 10 or 12 Gm. of PAS daily for at least three weeks prior to the drawing of the first blood samples; then Benemid was given along with the PAS for at least one week before blood samples were taken to determine the effect of the Benemid. Unless otherwise specified, the PAS was administered orally in the form of the sodium salt, as a 20 per cent aqueous solution flavord with peppermint, in four 3.0 Gm. doses daily—at 7:00 and 11:00 A.M. and 4:00 and 8:00 P.M. Liquid PAS was prepared fresh every other day by adding sufficient sodium bicarbonate to neutralize the PAS powder; the preparation was kept refrigerated in dark bottles, from which doses were dispensed to patients. Blood samples were usually drawn at one, four, and eleven hours after the last dose of PAS. One hour represents the approximate interval at which most patients show peak blood levels on non-enteric-coated PAS.[9, 10, 11, 13]

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Since four hours is the most usual interval between doses of PAS during the daytime in this hospital, the four-hour concentration is the usual minimum level to be expected during approximately sixteen hours of the day. The eleven-hour or fasting concentration is the minimum level for the twenty-four hours.

All subjects used in this study had pulmonary tuberculosis. Each patient was interviewed daily and a record kept of clinical symptoms of toxicity encountered.

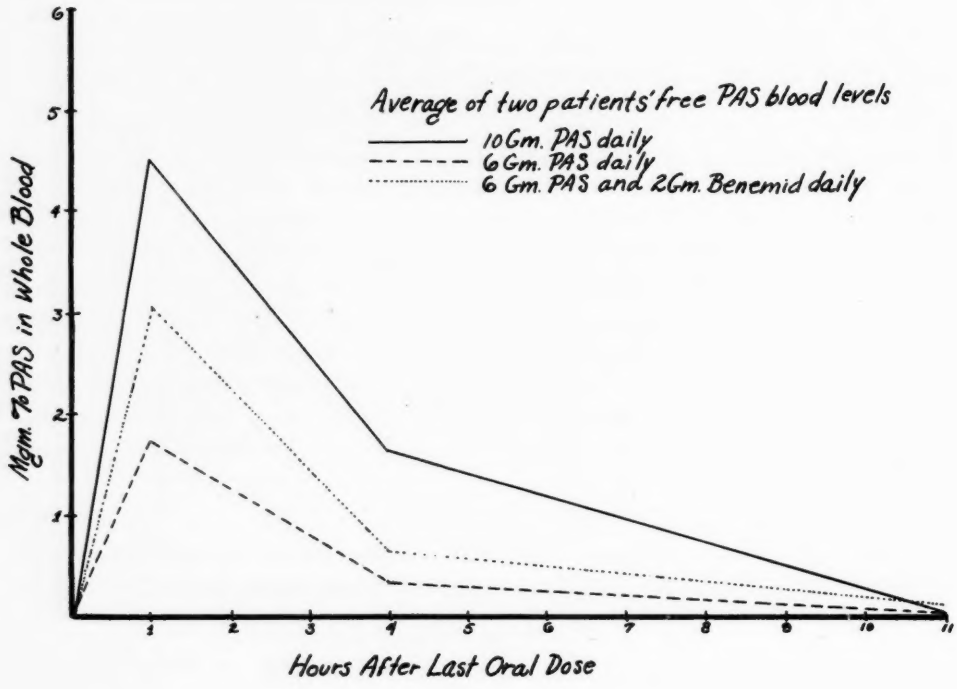
Average of two patients' free PAS blood levels
10 Gm. PAS daily
6 Gm. PAS daily
6 Gm. PAS and 2 Gm. Benemid daily
Mgm. % PAS in Whole Blood
Hours After Last Oral Dose

Fig. 1.

## RESULTS

Plasma levels of free aryl amine were found to be 30 to 40 per cent higher than whole blood levels run on the same specimens.

Table I shows the free aryl amine levels of six patients, all of whom received 12 Gm. of PAS daily (3.0 Gm. q.i.d.), to which 2 Gm. of Benemid (0.5 Gm. q.i.d.) were subsequently added. The blood concentrations at one and eleven hours were considerably higher on Benemid-PAS than on PAS alone; at four hours the Benemid-enhanced level was slightly lower than the "PAS only" level. These findings, except the eleven-hour concentration, are somewhat at variance with those of Boger and Pitts,[4] who found no significant difference in the one-half-hour levels, but an increasing differential in favor of Benemid at two, four, and eight hours.

Fig. 1 gives the average values of free blood aryl amine of two subjects who easily tolerated 6 Gm. of PAS daily (1.5 Gm. q.i.d.), but who complained of gastrointestinal symptoms on 10 Gm. per day (2.5 Gm. q.i.d.). Their blood concentrations were determined (a) while they received 10 Gm. daily, (b) one

week after the dose had been reduced to 6 Gm. daily, and (c) one week after Benemid (0.5 Gm. q.i.d.) had been added to the 6 Gm. daily dose of PAS. The addition of Benemid under these conditions approximately doubled the one- and four-hour blood PAS levels and left a faint trace at eleven hours, but none of these except the eleven-hour concentration was higher than that achieved by the administration of 10 Gm. of PAS alone. A comparison of Figs. 1 and 2 reveals that 2 Gm. of Benemid daily caused a proportionally greater elevation of blood PAS levels on 6 Gm. of PAS daily than on 12 Gm., although the actual increase was slightly greater on the larger dosage.

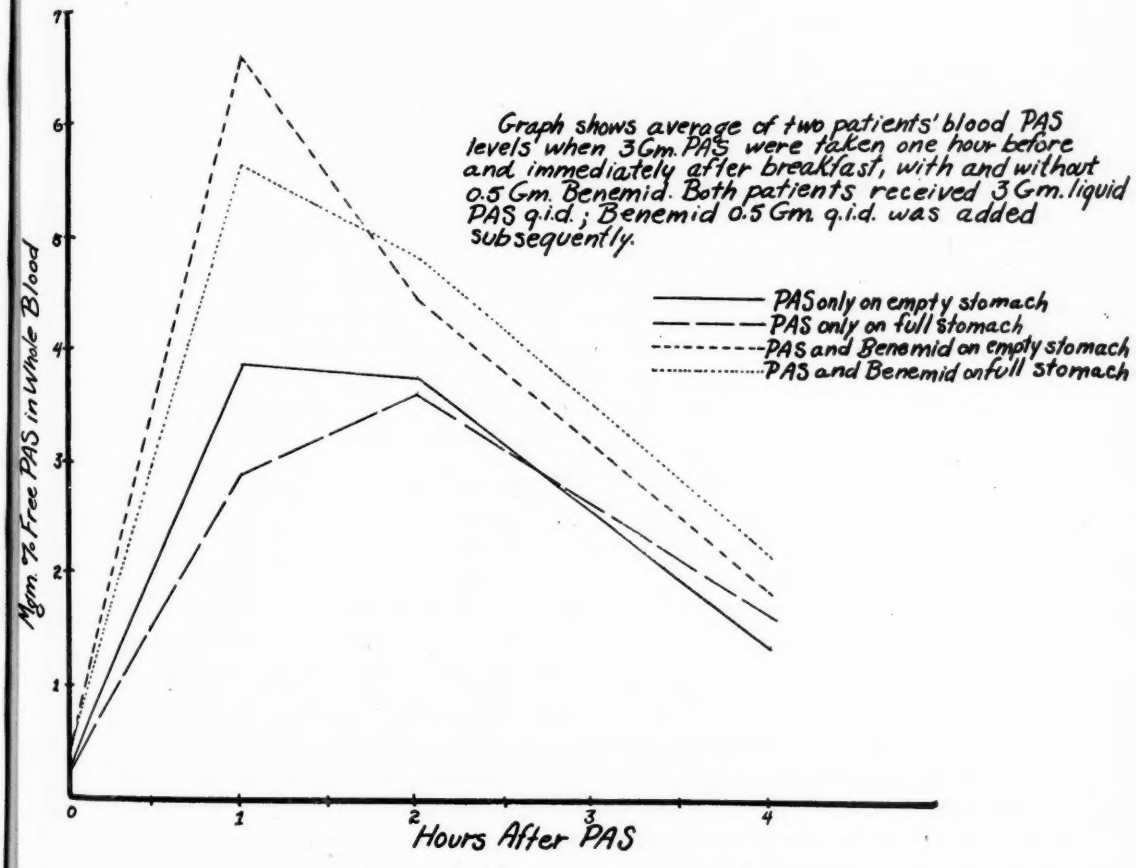
Graph shows average of two patients' blood PAS levels when 3 Gm. PAS were taken one hour before and immediately after breakfast, with and without 0.5 Gm. Benemid. Both patients received 3 Gm. liquid PAS q.i.d.; Benemid 0.5 Gm. q.i.d. was added subsequently.
PAS only on empty stomach
PAS only on full stomach
PAS and Benemid on empty stomach
PAS and Benemid on full stomach
Mgm. % Free PAS in Whole Blood
Hours After PAS
0
1
2
3
4
1
2
3
4
6
7

Fig. 2.

Fig. 2 shows Benemid virtually doubling the one-hour blood PAS levels, both when the PAS and Benemid were taken on an empty stomach and when they were taken immediately after a meal (breakfast); the two- and four-hour concentrations were increased by about one-third over those obtained with PAS alone. More sustained blood levels were produced by giving PAS immediately after meals than on a fasting stomach. PAS plus Benemid given after the meal produced the highest blood levels at two and four hours.

TABLE I. RESULTS OF 43 DETERMINATIONS ON 6 PATIENTS. ALL PATIENTS FIRST RECEIVED 12 GM. LIQUID PAS DAILY (3.0 GM. Q.I.D.); SUBSEQUENTLY 2 GM. BENEMID (0.5 GM. Q.I.D. WITH PAS) WERE ADDED

| HOURS AFTER MEDICATION | MEDICATION | BLOOD LEVELS OF FREE PAS (MG. %) | | | | | | | | AVERAGE |
|---|---|---|---|---|---|---|---|---|---|---|
| | | S. L. | A. R. | G. W. | W. S. | W. S. | D. H. | D. H. | W. R. | |
| 1 | PAS | 4.2 | 4.6 | 3.1 | 5.8 | 2.2 | 1.9 | 3.6 | 3.9 | 3.66 |
| | PAS and Benemid | 7.0 | 1.7 | 3.3 | 5.5 | 6.4 | 7.7 | 4.9 | 7.2 | 5.46 |
| 4 | PAS | 2.8 | 1.0 | 1.0 | 0.6 | 1.9 | 2.1 | 1.3 | | 1.53 |
| | PAS and Benemid | 1.0 | 0.4 | 0.9 | 0.9 | 1.6 | 2.7 | 2.7 | 0.9 | 1.39 |
| 11 | PAS | 0.2 | 0.0 | Tr. | 0.2 | | 0.3 | | 0.0 | 0.117 |
| | PAS and Benemid | 0.3 | 0.0 | Tr. | 0.4 | | 0.3 | | 0.1 | 0.183 |

Boger and Pitts,[4] by giving 4.0 Gm. of PAS to each of seven subjects who had received Benemid 0.5 Gm. every six hours for twenty-four hours, obtained plasma free aryl amine concentrations averaging 9.69 and 11.56 mg. per cent at one-half and two hours respectively. Three of our patients took 4.0 Gm. of PAS and 2.0 Gm. of Benemid on a fasting stomach after they had been receiving 3.0 Gm. of PAS and 0.5 Gm. of Benemid four times daily for two weeks. The average whole blood levels at one and two hours were 11.6 and 10.1 mg. per cent respectively. The highest individual level, 17.4 mg. per cent, occurred in a young woman weighing 96 pounds; she complained of anorexia, epigastric distress, and mild headache for eighteen hours following the medication. These are very high concentrations, especially when thought of in terms of their plasma equivalents, which are 30 to 40 per cent higher. However, in view of the percentage of patients who develop gastrointestinal symptoms on 4 Gm. Benemid plus 12 Gm. PAS daily, as is noted below in the discussion of toxicity, we feel that the information obtained on the foregoing dosage is not as valuable as that gained through the use of more easily tolerated quantities of both agents. Since administration of PAS to patients four times daily presented no particular problem, and since a large percentage of our patients tolerated no more than 12 Gm. of PAS daily, we conducted our studies mostly on patients receiving 12 Gm. or less of PAS and 2 Gm. of Benemid per day.

Two patients whose average weight was 206 pounds had average blood levels of 3.85 and 1.0 mg. per cent at one and four hours respectively, compared to 5.27 and 1.43 mg. per cent for three patients whose weights averaged 129 pounds. The heavier subjects had only slight traces of free aryl amine in the fasting blood, while the lighter ones had an average level of 0.13 mg. per cent. This relation between body weight and blood concentration of PAS, noted before,[1] seems to indicate the desirability of body weight, as well as individual tolerance, being a factor in the choice of PAS dosage for each patient.

Table II gives the results of free and total aryl amine determinations on the whole blood of eight patients. The difference between free and total values represents the acetylated fraction. This fraction was fairly constant in some patients (F. Z., B. E., and A. R.) regardless of the total aryl amine con-

TABLE II. FIGURES IN COLUMNS UNDER INITIALS OF PATIENTS ARE WHOLE BLOOD CONCENTRATIONS OF ARYL AMINE IN MILLIGRAMS PER 100 ML. PATIENTS B. E. AND F. M. RECEIVED 6 GM. PAS DAILY; THE OTHERS RECEIVED 12 GM. PERCENTAGE OF ACETYLATED AMINE EQUALS $\frac{\text{TOTAL AMINE MINUS FREE AMINE}}{\text{TOTAL AMINE}} \times 100$

| MEDICATION | HOURS AFTER PAS | | W. S. | D. H. | F. Z. | B. E. | S. L. | G. W. | A. R. | F. M. | AVERAGE PER CENT ACETYLATED AMINE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PAS only | 1 | Free | 5.8 | 1.9 | 4.2 | 1.7 | 4.2 | 3.1 | 4.6 | 1.8 | 11.4 |
| | | Total | 6.3 | 2.1 | 4.8 | 2.0 | 4.5 | 3.6 | 4.8 | 2.3 | |
| | 4 | Free | 0.6 | 2.1 | 3.4 | 0.3 | 2.8 | 1.0 | 1.0 | 0.4 | 19.8 |
| | | Total | 1.0 | 2.3 | 3.8 | 0.5 | 2.8 | 1.1 | 1.2 | 0.6 | |
| | 11 | Free | 0.2 | 0.3 | 4.6 | 0.0 | 0.2 | Tr. | Tr. | 0.0 | 26.5 |
| | | Total | 0.3 | 0.5 | 4.9 | 0.0 | -- | 0.1 | Tr. | 0.0 | |
| PAS plus Benemid | 1 | Free | 5.5 | 7.7 | 4.9 | 2.5 | 7.0 | 3.3 | 4.7 | 3.7 | 17.2 |
| | | Total | 6.3 | 8.3 | 6.0 | 3.4 | 7.7 | 4.8 | 5.1 | 4.9 | |
| | 4 | Free | 0.9 | 2.7 | 3.7 | 0.5 | 1.0 | 0.9 | 1.8 | 0.8 | 31.6 |
| | | Total | 2.1 | 2.8 | 4.9 | 1.4 | 1.6 | 1.3 | 2.4 | 1.9 | |
| | 11 | Free | 0.4 | 0.3 | 2.8 | Tr. | 0.3 | Tr. | 0.2 | Tr. | 47.7 |
| | | Total | 0.7 | 0.8 | 3.8 | 0.2 | 0.5 | 0.2 | 0.6 | 0.3 | |

centrati n, and quite variable in others; it constituted the highest percentage of the total in the two (B. E. and A. R.) taking the smallest amount of PAS—6 Gm. daily.

The decrease in concentration of acetylated amine with time after the last dose of PAS was proportionally less than the decrease in the total amine. This accounts for the percentages of acetylated amine being higher at four and eleven hours, although in some cases the actual concentrations were the same as, or lower than, those at one hour. In nearly every patient, the addition of Benemid caused an increase in the actual and proportional amounts of acetylated amine. Benemid, by interfering specifically with the glycine conjugation of PAS, may, as has been suggested,[5] force more PAS into other channels of conjugation, including acetylation, thus accounting for the increase in acetylated amine during its administration.

## TOXICITY

Twelve patients had Benemid, 0.5 Gm. q.i.d., added to their regular 12 Gm. daily dosage of PAS. Of this group, four or 33⅓ per cent experienced symptoms when the Benemid was added. Seven of those who remained symptom free on 2 Gm. Benemid and 12 Gm. PAS daily subsequently had their Benemid increased to 4 Gm. (1 Gm. q.i.d.); five or 71.4 per cent of this group quickly developed symptoms on the increased dose. These patients could tolerate 4 Gm. of Benemid *or* 12 Gm. of PAS daily, but encountered difficulty when both were taken simultaneously. Symptoms were most likely not due to high blood levels of PAS, for a blood concentration of 40 mg. per cent is said to be necessary to produce symptoms,[2] and that level was not attained. Inasmuch as the incidence of toxicity in our series is higher than that previously reported, it is possible that impurities in the PAS administered could have been a factor. Symptoms, in order of frequency of occurrence, were nausea, epigastric distress, vomiting, headache, and malaise. The only two patients who tolerated 4 Gm. of Benemid and 12 Gm. of PAS daily both weighed more than 200 pounds. One

patient developed severe vomiting on two different occasions within twenty-four hours after the addition of 0.5 Gm. Benemid q.i.d. to the PAS. This patient, while tolerating 12 Gm. of PAS daily quite well, was a very tense individual who also experienced abdominal discomfort after taking small doses of aspirin and Pyribenzamine. The two patients who failed to tolerate 10 Gm. of PAS daily did very well on 6 Gm. of PAS plus 2 Gm. of Benemid per day.

## COMMENT

There is some indication that during the simultaneous administration of PAS and streptomycin, the rate of emergence of streptomycin resistance is lower with the larger dosages of PAS.[16] If the mean blood free PAS level, and not the total amount of PAS absorbed, is the important factor in the effectiveness of oral PAS, then Benemid, by virtually doubling the one- and four-hour levels of patients on a low dosage of PAS, should be valuable in preventing development of streptomycin resistance of these patients' organisms.

Concentrations of PAS required to suppress growth of human tubercle bacilli in vitro have been reported as 0.156 mg. per cent,[18] 1 mg. per cent,[8] 20 mg. per cent,[7] and up to 100 mg. per cent,[14] although most strains appear to be sensitive to 1 mg. per cent, which is the level above which organisms are arbitrarily said to be "resistant." In our experience Benemid in the dosage employed produced a fasting blood PAS level of 1 mg. per cent in only one of twelve patients receiving 3 Gm. sodium PAS four times daily.

In two of our patients receiving 10 and 12 Gm. of PAS in tablet form daily, the minimum blood free aryl amine level was 0.1 mg. per cent for one and 3.4 mg. per cent for the other. Dye[9] reported that a daily dosage of 10.8 Gm. or more of liquid sodium PAS divided into equal doses given orally every three hours maintained, on the average, free plasma PAS levels of 1.0 mg. per cent after a week of treatment. These findings, together with the experience of Swedish investigators with various preparations including enteric-coated granules of PAS,[1, 13] suggest that perhaps much can be accomplished in maintaining high blood levels by manipulation of the frequency and form of PAS dosage before Benemid is added to the regime. Benemid should be especially useful in producing higher mean PAS blood concentrations in those patients who tolerate only low PAS dosages and/or the liquid preparation only. However, the status of Benemid in PAS therapy cannot be satisfactorily delineated until it has been determined whether or not continuous maintenance of certain blood levels is more effective therapeutically than are the same levels achieved intermittently.

## CONCLUSIONS

Benemid substantially elevates blood free PAS levels, the increase being most remarkable several hours after the last oral dose of PAS; but it rarely produces a fasting blood PAS level of 1 mg. per cent under conditions of this experiment.

Benemid causes a greater percentage increase in blood free aryl amines in patients receiving 6 Gm. of PAS daily than in those receiving 12 grams.

Benemid causes an increase in the relative and actual amounts of acetylated amine in the blood.

A higher percentage of patients develops symptoms of toxicity, chiefly gastrointestinal, on a combination of PAS and Benemid than on PAS alone; but Benemid in dosages of 2 or 4 Gm. daily produces no symptoms when given alone, i.e., without PAS.

Manipulation of the form and frequency of the PAS dose should not be neglected as a means of maintaining desired blood PAS levels.

The routine use of Benemid with PAS cannot be recommended from this data although its value is highly suggestive.

The author wishes to thank Dr. Allan Hurst, former Medical Director of National Jewish Hospital at Denver, Colo., and William E. Dye, 1st Lt. U.S.A.F. (M.S.C.), Fitzsimons Army Hospital, Denver, Colo., for their generous assistance in this work.

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# SERUM CONCENTRATIONS OF CHLORAMPHENICOL FOLLOWING INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION

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CHLORAMPHENICOL has become established as a potent antibiotic. Therapeutic concentrations can be maintained with oral administration, and toxicity is of low order. As with all antibiotics, a parenteral form is needed for patients unable to tolerate oral medication. However, chloramphenicol is insoluble in aqueous media because there are neither acidic nor basic groups capable of forming salts. This difficulty has been recently solved by the development of a formula containing 25 per cent chloramphenicol in 50 per cent aqueous acetyl dimethylamine.* The present study was undertaken to test the toxicity of this new preparation, and to measure serum concentrations of chloramphenicol following parenteral administration.

## MATERIALS AND METHODS

For a period of three months all adult patients with bacterial pneumonia, lung abscess, or bronchiectasis entering the King County Hospital were treated with intravenous chloramphenicol. Two dosage regimens were used. The first group (thirty patients) received 0.5 Gm. every twelve hours. In the other group (fifteen patients) 1.0 Gm. was administered once daily. Serum levels were measured at one, three, six, twelve, and twenty-four hours following an injection. Although chloramphenicol is stable, serum was not allowed to stand for more than twelve hours without freezing. Assays were performed by a tube dilution method, with inhibition of *B. cereus* at four hours as the end point.

Some of the patients were given chloramphenicol intravenously for three or four days, and then received the antibiotic by mouth, to allow a comparison of the serum levels obtained by the two routes of administration.

In a group of six patients serum levels were measured after intramuscular administration of the chloramphenicol solution.

## RESULTS

*Toxicity.*—The drug was supplied in liquid form in 2 c.c. ampules, each containing 0.5 Gm. of chloramphenicol. When this concentrated form was administered intravenously over a period of two to three minutes, five of ten patients complained of pain along the course of the vein, and in six patients the veins subsequently thrombosed. A fall in blood pressure was encountered in three of ten patients. In one of these, the hypotension was accompanied by a chill with cyanosis, and the temperature rose to 39° C. for two hours.

Because of these difficulties, the concentrated solution was diluted in at least 100 c.c. of saline or glucose solution. Because of a tendency to precipita-

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*The Chloromycetin parenteral was kindly supplied by Dr. E. C. Vonder Heide of Parke, Davis & Company, Detroit 32, Mich.

tion, it was necessary to withdraw the contents of the ampule in a dry syringe, and then inject it rapidly beneath the surface of the diluting fluid.*

Forty-five patients in this study received the chloramphenicol diluted according to the above precautions and administered by intravenous drip over periods of fifteen to thirty minutes. Very few toxic reactions were observed. In one instance, administration was followed by a temperature rise to 38° C. for two hours. In two patients who received intravenous injections twice daily, the antecubital veins became thrombosed after six days.

The six patients receiving 1.0 Gm. intramuscularly tolerated the preparation well, and did not complain of pain at the site of injection.

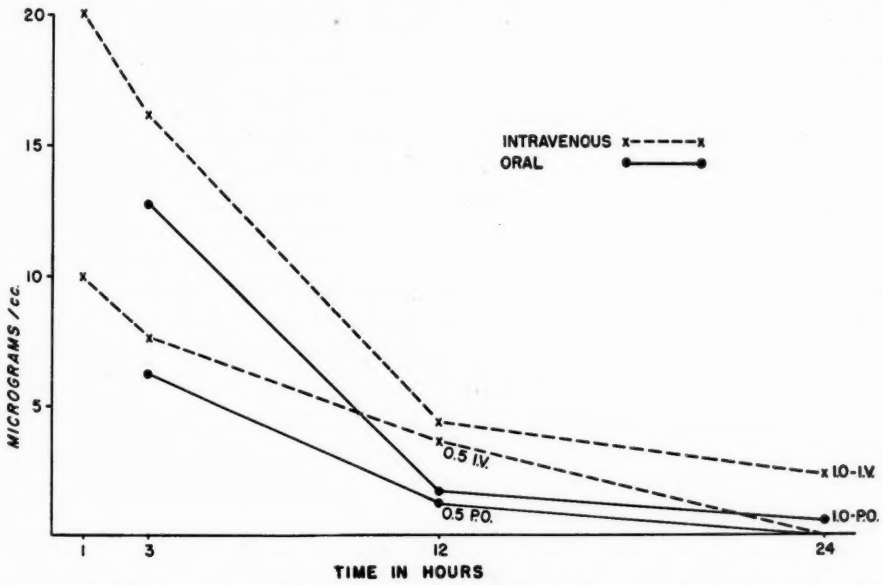


Fig. 1.—Average serum levels after intravenous and oral administration of 0.5 Gm. and 1.0 Gm. of chloramphenicol.

*Serum Levels Following Intravenous Administration of Chloramphenicol.*—Serum levels following single intravenous injections of 0.5 Gm. were measured in thirty patients as shown in Table I. At three hours, almost all serum levels were either 5 or 10 μg. per cubic centimeter, with an average of 7.66 μg. per cubic centimeter. At twelve hours the average level had fallen to 3.84, with only 73 per cent still having a measurable level (at least 2.5 μg./c.c.). There was no measurable level at twenty-four hours in three patients.

Serum levels following 1.0 Gm. intravenously were measured in fifteen patients (Table I). At three hours, the average level was approximately twice as high (16.1 μg./c.c.) as that following 0.5 Gm. At twelve hours, the average levels with both amounts were approximately 4 μg. per centimeter, but 90 per cent had measurable levels following 1.0 Gm. as compared with 73 per cent following 0.5 Gm. At twenty-four hours following 1.0 Gm. measurable levels were still present in 87 per cent of the patients.

*As per directions of Parke, Davis, and Company.

TABLE I. SERUM CHLORAMPHENICOL LEVELS FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION OF 0.5 AND 1.0 GRAMS

| | HOURS AFTER INJECTION | CHLORAMPHENICOL 0.5 GM. | | CHLORAMPHENICOL 1.0 GM. | |
|---|---|---|---|---|---|
| | | NUMBER OF DETERMINATIONS | AVERAGE LEVEL (μG./C.C.) | NUMBER OF DETERMINATIONS | AVERAGE LEVEL (μG./C.C.) |
| Administered intravenously | 1 | 2 | 10 | 4 | 20 |
| | 3 | 16 | 7.7 | 17 | 16.1 |
| | 6 | 9 | 5.0 | 2 | 7.5 |
| | 12 | 85 | 3.8 | 21 | 4.2 |
| | 24 | 3 | 0 | 22 | 2.4 |
| Administered orally | 1 | - | - | 5 | 23 |
| | 3 | 12 | 6.3 | 20 | 12.7 |
| | 6 | 3 | 4.2 | 4 | 11.2 |
| | 12 | 17 | 1.3 | 12 | 1.9 |
| | 24 | 3 | 0 | 8 | 0.6 |

A group of seventeen patients, who had received 0.5 Gm. intravenously twice daily for three or four days, was given 0.5 Gm. orally (Fig. 1). Compared with the intravenous levels, the oral levels varied from slightly lower at three hours to approximately 50 per cent lower at twelve hours. In the group treated orally only 41 per cent had measurable levels at twelve hours, as compared with 73 per cent in the group treated intravenously.

After receiving 1.0 Gm. daily intravenously for three or four days, ten patients received 1.0 Gm. by mouth in a single dose for a day or two. Compared to levels following intravenous administration of 1.0 Gm., levels with the oral route were slightly lower at three hours, and about 50 per cent lower at twelve hours (Fig. 1). As with 0.5 Gm., the levels tended to persist for a longer time following the intravenous route. At twelve hours, 90 per cent had measurable levels as compared with 67 per cent following oral administration; at twenty-four hours, the ratio was 87 per cent following intravenous to 12 per cent following oral administration.

*Serum Levels Following Intramuscular Administration of Chloramphenicol.*—Six patients received the parenteral preparation intramuscularly. In four, serum levels following 0.5 Gm. of chloramphenicol were measured at one and three hours after a single injection. At one hour, three levels were 2.5 μg. per cubic centimeter, and in the fourth there was no measurable level. At three hours, the levels were also 2.5 μg. per cubic centimeter.

Two patients were given 0.5 Gm. every six hours for twenty-four hours. Serum levels were measured three hours after each injection. In both patients levels varied from 2.5 to 5.0 μg. per cubic centimeter in random manner, indicating that there was no cumulative effect.

## DISCUSSION

The foregoing data indicate that the intravenous administration of a new parenteral preparation of chloramphenicol provides effective therapeutic concentrations when oral medication is not feasible. The intravenous administration of 0.5 Gm. and 1.0 Gm. of chloramphenicol is followed by measurable blood levels for twelve and twenty-four hours, respectively. At three hours, levels were only slightly higher than those obtained following oral administration. However, at twelve and twenty-four hours the concentrations following

intravenous administration were approximately twice as high as following equal doses orally. This tendency to parallelism of levels following the two routes of administration is indicative of fairly complete absorption of chloramphenicol from the intestinal tract, and differs from the results obtained with aureomycin. With aureomycin the levels obtained following intravenous administration are from two to ten times higher than following oral administration.[1, 2] Terramycin appears to be intermediate, with the differential being about two to four times higher following intravenous therapy.[3]

In the treatment of severe infections, serum chloramphenicol concentrations of 10 to 20 μg. per cubic centimeter are considered desirable. The present data indicate that these requirements will be met by 1.0 Gm. intravenously every twelve hours. Doses of this magnitude were well tolerated when the concentrated chloramphenicol solution was diluted in 100 c.c. or more of glucose or saline solution.

The chloramphenicol solution was also well tolerated when injected intramuscularly in the undiluted form, but the serum concentrations obtained with this route were clearly not in the therapeutic range.

## SUMMARY

In fifty patients serum chloramphenicol levels were determined following the intravenous administration of a new parenteral preparation. Following a single injection of 1.0 Gm., serum concentrations averaged 20 μg. per cubic centimeter at one hour, and gradually declined to an average of 2.4 μg. per cubic centimeter at twenty-four hours. Following 0.5 Gm. intravenously, the serum concentrations fell from an average of 10 μg. per cubic centimeter at one hour to 3.8 μg. per cubic centimeter at twelve hours.

When levels following oral and intravenous administration were compared, it was found that serum concentrations were slightly higher with the intravenous route at three hours, but were twice as high at twelve hours, and persisted for a longer period than with the oral route.

From the data obtained, it would appear that when chloramphenicol is indicated, and oral administration is not feasible, 1.0 Gm. every twelve hours intravenously will provide effective therapy.

The parenteral preparation was also well tolerated by the intramuscular route, but did not give serum levels adequate for the therapy of most infections.

We are indebted to Miss Betty O'Leary for technical assistance.

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# ACTION OF A SYNTHETIC ATROPINE SUBSTITUTE IN NORMAL INDIVIDUALS

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THIS report is a summary of studies undertaken to evaluate the effect of a,a-diphenyl-γ-dimethyl aminovaleramide hydrogen sulfate (hereafter referred to as "BL-139") on certain phases of fasting gastric secretion and gastric and small bowel motility. This drug is a parasympatholytic agent, and its activity has been compared with that of atropine sulfate in a group of normal individuals. Its structure is given in Fig. 1, with that of atropine for comparison. It is of interest that BL-139 is not an organic acid ester as is the case with most synthetic atrophine substitutes.

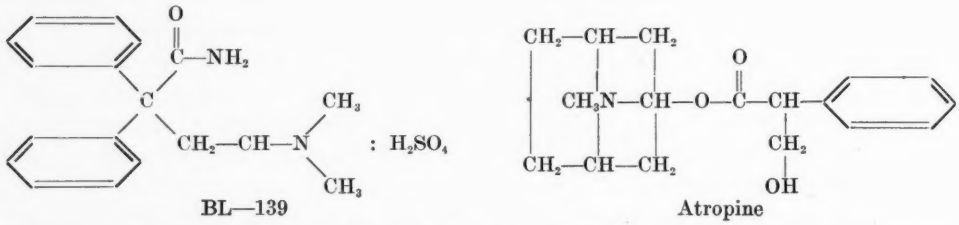


Fig. 1.—Structural formula of a,a-diphenyl-γ-dimethyl aminovaleramide hydrogen sulfate, compared with atropine.

## METHODS AND RESULTS

*Effect on the Volume, Free Hydrochloric Acid, and pH of Fasting Gastric Secretion.*—Subjects consisted of a group of normal college students studied after a twelve-hour fast by means of intubation with measurement of gastric secretory volume, free hydrochloric acid, and pH, as well as simultaneous kymographic record of gastric motility by intragastric balloon. Comparison was made of the effect of subcutaneous injections of saline, atropine sulfate 0.6 mg., atropine sulfate 1.0 mg., BL-139 1.0 mg., and BL-139 3.0 milligrams. Results are shown in Figs. 2, 3, and 4, representing the average of individual studies as indicated.

*Volume:* Atropine 0.6 mg. and BL-139 1.0 mg. produce a significant and almost identical decrease in gastric secretory volume, when compared with saline controls. Atropine 1.0 mg. and BL-139 3.0 mg. produce a slightly greater depression of secretion.

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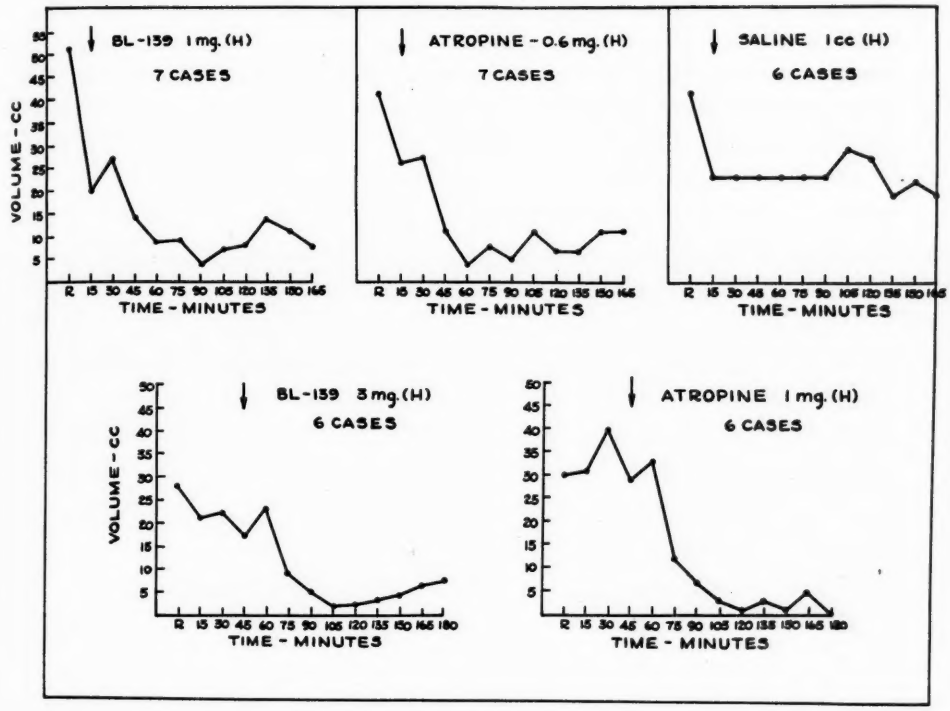


Fig. 2.—Volume in cubic centimeters of average continuous fasting gastric secretion, showing effect of hypodermic injection of 1 c.c. saline, 0.6 mg. atropine sulfate, 1 mg. atropine sulfate, 1 mg. BL-139, and 3 mg. BL-139.

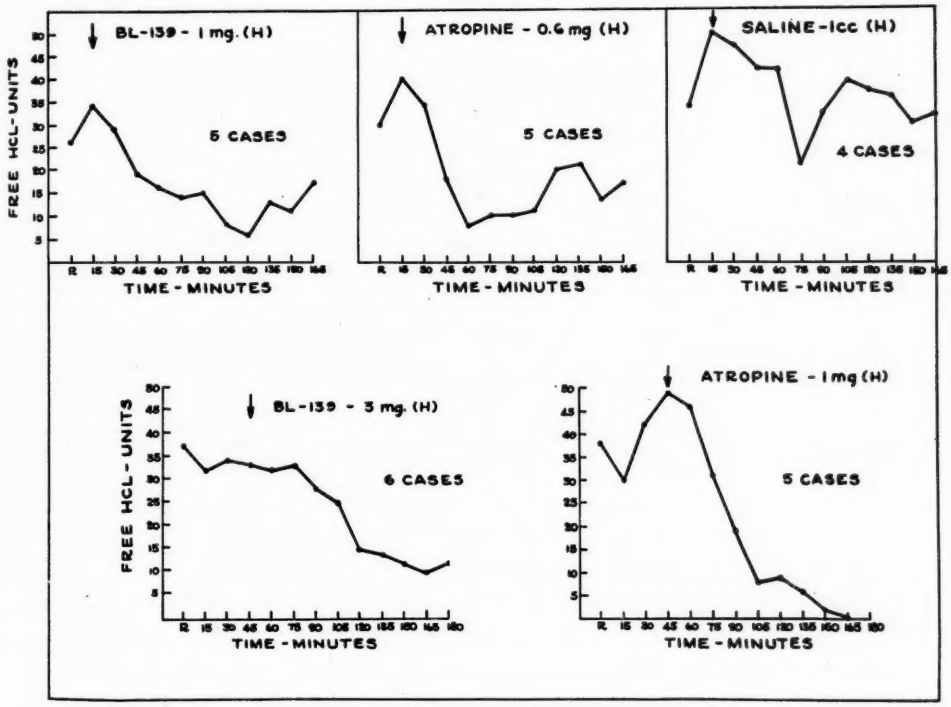


Fig. 3.—Units of free hydrochloride acid of average continuous fasting gastric secretion, showing effect of injections of saline, atropine sulfate, and BL-139.

*Free Hydrochloric Acid:* Atropine 0.6 mg. and BL-139 1.0 mg. again produce a similar decrease in the output of free hydrochloric acid. However, atropine 1.0 mg. appears more effective in this respect than BL-139 in a dosage of 3.0 mg. or 1.0 milligram.

*pH:* In its effect on pH, atropine in a dosage of 0.6 mg. or 1.0 mg. appears slightly more effective than BL-139 in a dose of 1.0 mg. or 3.0 milligrams.

*Effect on Gastric and Small Bowel Motility.*—Gastric motility was measured by intragastric balloon in all patients subjected to gastric secretion studies. Both BL-139 and atropine produced moderate but not complete or sustained inhibition of gastric motility in the doses used.

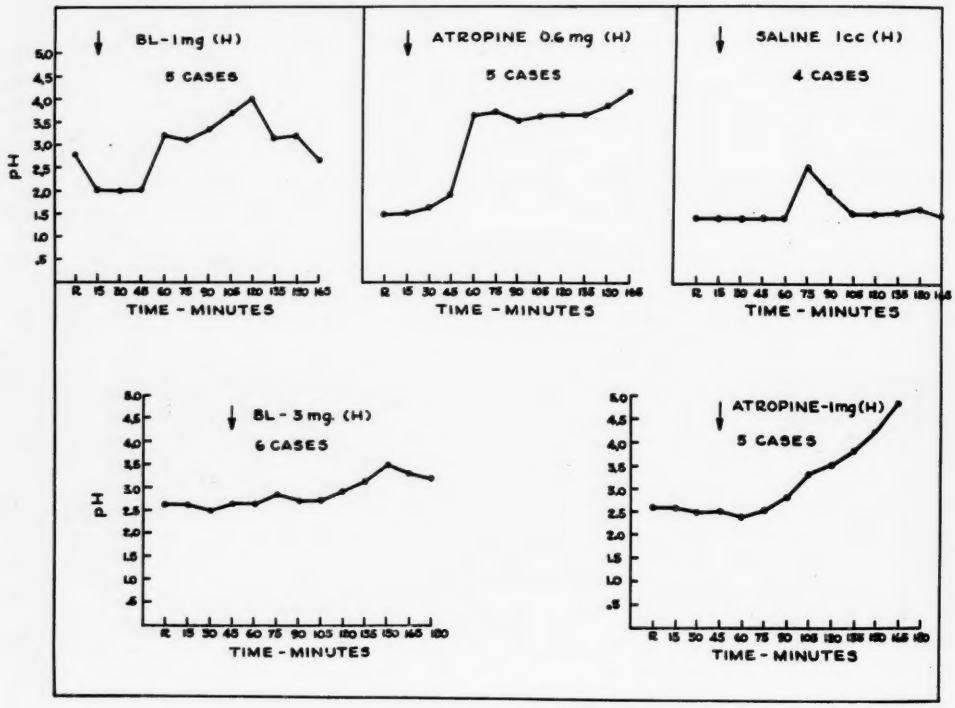


Fig. 4.—pH of average continuous fasting gastric secretion, showing effect of injections of saline, atropine sulfate, and BL-139.

To obtain a more easily measured index of the activity of these drugs on upper digestive tract motility, a group of normal individuals was subjected to routine roentgenologic examination of the upper gastrointestinal tract, including hourly films for five hours after ingestion of barium. As arbitrary indices of motility, the time of complete gastric emptying, the time at which the head of the barium column entered the colon, and the time at which the tail of the barium column was collected in the right lower quadrant of the abdomen were noted. All of these subjects were examined in this fashion as saline controls, with atropine 0.6 mg. (hypodermic), and BL-139, 1 or 2 mg. (hypodermic). The results are summarized in Table I.

Gastric motility, as measured by the time of gastric emptying, is markedly inhibited by these drugs, the average of saline controls being 2.9 hours, that of atropine (0.6 mg.) being +4.4 hours, and that of BL-139 being +4.2 hours. The difference between the effect of doses of 1 mg. and 2 mg. of BL-139 on motility was not significant. Similarly, these drugs produced a marked inhibition of

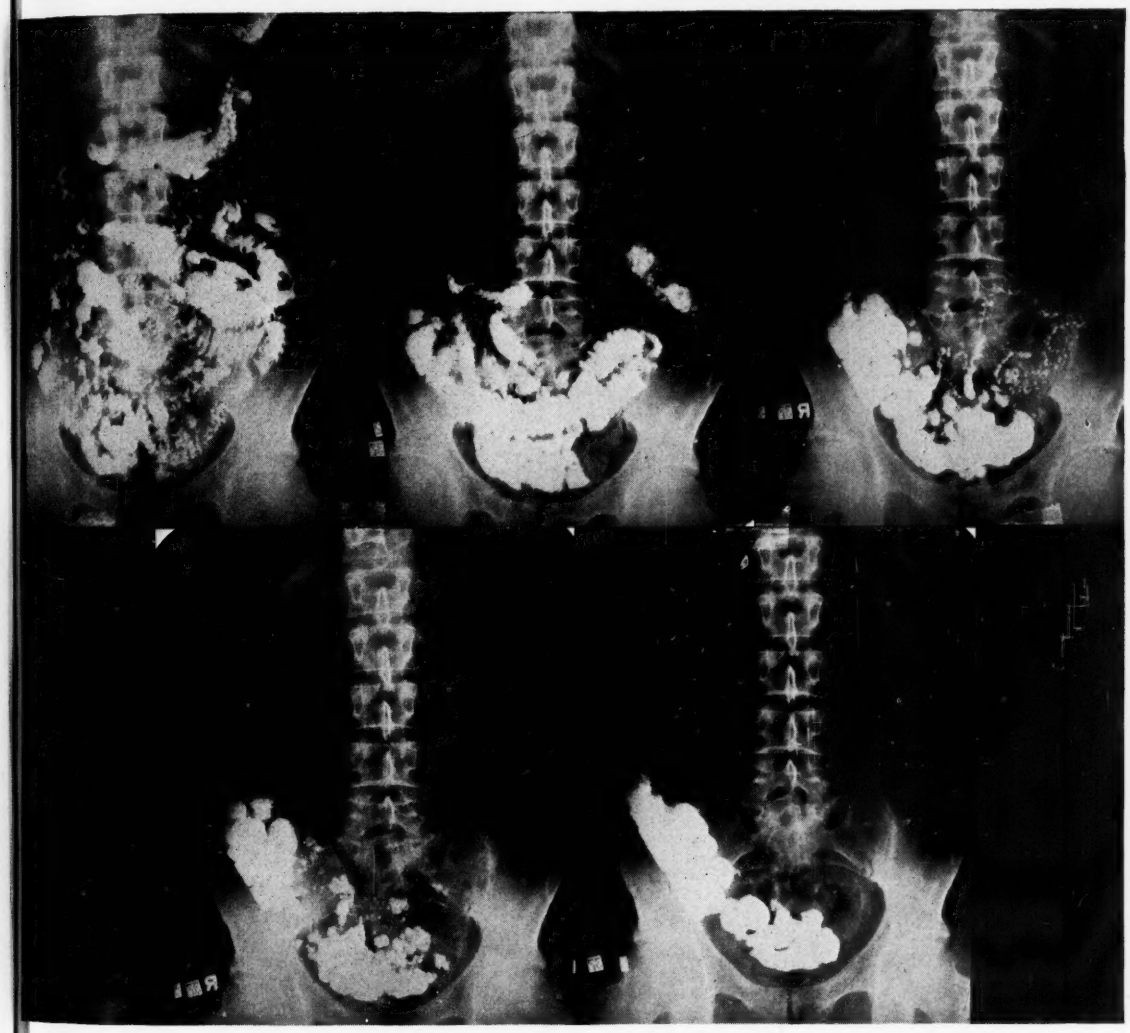

Fig. 5.—Normal upper gastrointestinal tract examination of 30-year-old man, following injection of 0.5 c.c. saline (control). Films are taken at one, two, three, four and five hours, and show gastric emptying at two hours, with the head of the barium column in the colon at three hours, and the tail of the column in the right lower quadrant at four hours.

small bowel motility, as measured by the transit time of the barium column. In the saline controls, the head of the barium column reached the right colon in an average period of 2.27 hours; with atropine, this progress required an average of +4.3 hours; and with BL-139, an average of +4.8 hours. In the saline controls the tail of the barium column was concentrated in the right lower quadrant in

an average period of 4.18 hours; with both atropine and BL-139, this progress required an average period of more than 5.0 hours. Thus, these drugs produce a definite inhibition in upper gastrointestinal tract motility. In the doses used, atropine and BL-139 produce a similar degree of effect. Figs. 5, 6, and 7 demonstrate these motility changes in the same subject, studied after saline control, atropine, and BL-139 administration.

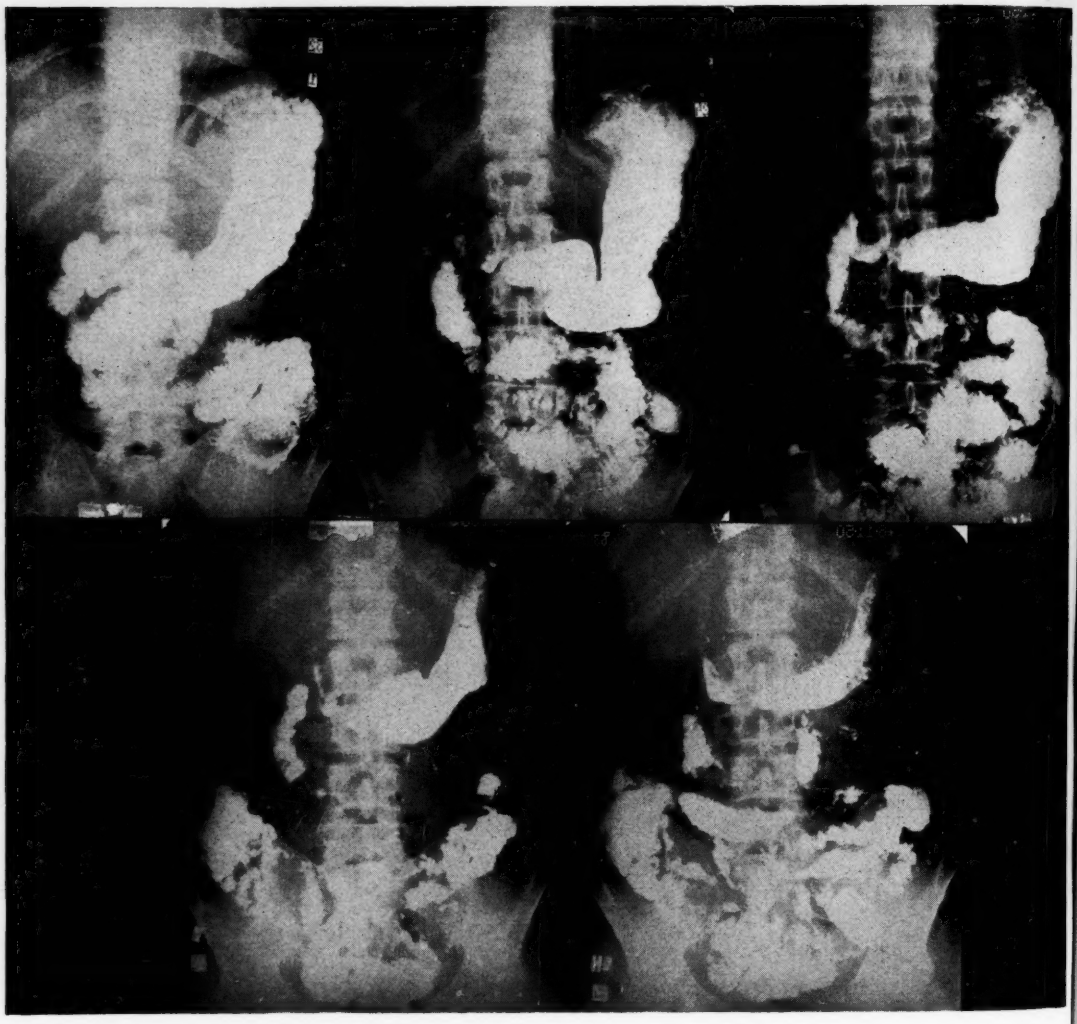

Fig. 6.—Same patient after injection of 0.6 mg. atropine sulfate, showing delayed gastric emptying, a slight decrease in small bowel transit time, and sufficient small intestine mucosal distortion to warrant a diagnosis of small bowel dysfunction. Films taken at one, two, three, four and five hours.

*Side Reactions and Dosage.*—Clinical side reactions of BL-139 are similar to those encountered with atropine, on the basis of observations during use of the drug in ninety-one random hospital patients. These side effects consist mainly of xerostomia and to a lesser extent mydriasis; they begin to appear when a

single oral dose exceeds 0.5 milligram. Since use of a drug of this type tends to be limited to a dosage which does not produce markedly disturbing side effects, it seems likely that a usually acceptable dose is 0.5 mg. given by mouth three times daily—making the drug comparable to atropine in dosage. As with atropine,

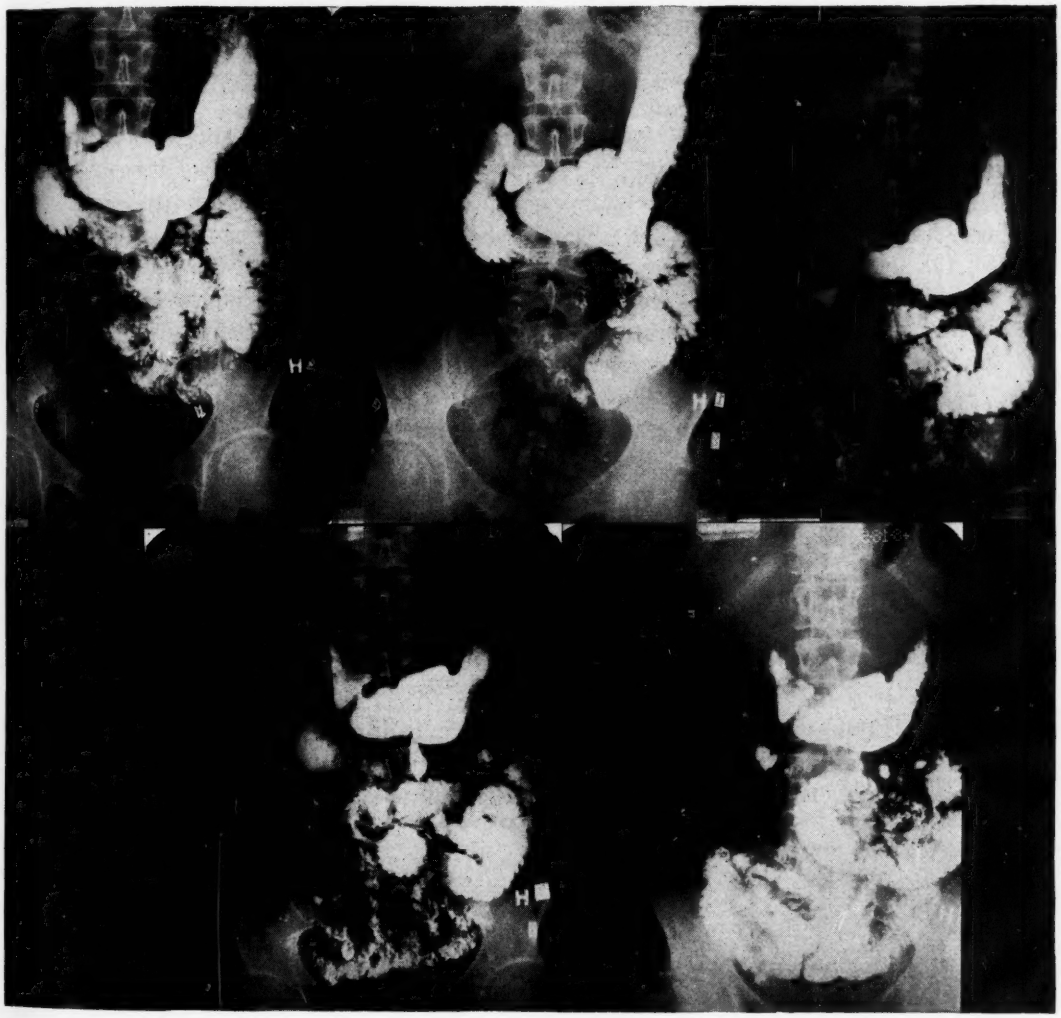

Fig 7.—Same patient after injection of 2 mg. BL-139, again showing delayed gastric emptying, and decreased small bowel transit time. Films taken at one, two, three, four, and five hours.

there is considerable individual variability in tolerance to larger doses. However, sufficiently distressing depression of salivary and lacrymal gland secretion occurs in a majority of patients receiving a total daily dose of 3.0 mg. or more to preclude use of the drug in doses larger than this under ordinary circumstances. One of the more disturbing side reactions at and above this dosage level is the frequent occurrence in male patients of urinary dribbling and even bladder distention.

TABLE I. SUMMARY OF EFFECT ON UPPER GASTROINTESTINAL TRACT MOTILITY: ROENTGENOLOGIC EXAMINATIONS

| | GASTRIC EMPTYING BY (HR.) | HEAD OF BARIUM COLUMN IN COLON AT (HR.) | TAIL OF BARIUM COLUMN IN R.L.Q. AT (HR.) |
|---|---|---|---|
| Controls: 10 patients, 11 examinations | 2.9 | 2.27 | 4.18 |
| Atropine: 10 patients, 0.6 mg. (H) | +4.4 | +4.3 | +5.0* |
| BL-139: 5 patients, 1 mg. (H) | +4.0 | +4.8 | +5.0 |
| 5 patients, 2 mg. (H) | +4.4 | +4.8 | +5.0 |
| Total, average | +4.2 | +4.8 | +5.0 |

*"Motor dysfunction of small bowel" induced in one case by atropine.

## SUMMARY

BL-139 is very similar to atropine in its effect on gastric secretory volume, free hydrochloric acid, and pH. The effect of 0.6 mg. atropine sulfate (H) and of 1.0 mg. BL-139 (H) in the same group of normal patients appears almost identical in effect on secretion. When 1.0 mg. of BL-139 is compared with 1.0 mg. of atropine sulfate, atropine has slightly greater maximal effect on free hydrochloric acid secretion and pH.

The effect of both BL-139 and atropine on gastrointestinal motility (gastric as measured by balloon studies and roentgenogram, and small bowel as measured by roentgenogram) appears similar—i.e., moderate but not complete or sustained inhibition of motility.

Clinical side reactions of BL-139 are similar to those of atropine.

The incidence of atropine-like side reactions with BL-139 suggests that its dosage in clinical use should be similar to that of atropine in order to avoid significant side reactions in a majority of patients (that is, average single dose of 0.5 mg. for both).

## CONCLUSION

BL-139 has an effect on gastric secretion and upper digestive tract motility which is very similar to the "parasympatholytic" activity of atropine. The available evidence does not indicate that BL-139 is superior to atropine.

# BIOCHEMICAL STUDIES ON LEUCOCYTES

## II. Phosphatase Activity in Chronic Lymphatic Leucemia, Acute Leucemia, and Miscellaneous Hematologic Conditions

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### INTRODUCTION

In a previous paper[1] it was shown that characteristic chemical enzymatic patterns may be described for the leucocytes of physiologic leucocytosis and chronic myelocytic leucemia. Data in the literature pertaining to leucocyte glycogen, zinc, histamine, –SH groups, and alkaline and acid phosphatase were reviewed, and it was emphasized that pronounced differences occur in the content of each of these constituents in the leucocytes of leucocytosis and chronic myelocytic leucemia. In this laboratory, it has been shown that neutrophilic leucocytosis is accompanied by extremely high alkaline phosphatase levels per leucocyte, normal acid phosphatase, and low histamine[2] values. Conversely, the leucocytes of chronic myelocytic leucemia contain strikingly low levels of alkaline phosphatase activity, normal to high acid phosphatase and high histamine values, the pattern of leucemia persisting throughout periods of clinical remission induced by therapy. It was concluded in these studies that the alkaline phosphatase resides chiefly in the cells of the granulocytic series and that the acid phosphatase is probably distributed in a fairly uniform manner throughout both the lymphatic and granulocytic cells.

It is the purpose of this report to record the alkaline and acid phosphatase values in groups of patients with chronic lymphatic leucemia, acute leucemia, and a variety of miscellaneous hematologic disorders including polycythemia vera. The methods used are the same as those described in the previous report,[1] consisting essentially of isolation of leucocytes by the fibrinogen method[3] and determination of phosphatase activity on saline suspensions of cells using saponin as a surface-active agent and sodium $\beta$-glycerophosphate as the substrate. Alkaline phosphatase activity was determined at a pH of 9.9, and acid phosphatase at pH 5.0.

### RESULTS

The data are presented in Fig. 1 and Tables I, II, and III. Fig. 1 is a scattergram which depicts the individual determinations of alkaline and acid phosphatase in chronic lymphatic leucemia and acute leucemia in contrast to those in normal individuals.

*Normal Subjects.*—The normal group consists of the twenty-three individuals described in the first paper. As shown in Fig. 1, the alkaline phosphatase levels (expressed in terms of milligrams of phosphorus liberated per $10^{10}$ leucocytes) ranged from 13.4 to 58.0 with a mean of 25.8. The acid phosphatase

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TABLE I. PHOSPHATASE ACTIVITY IN INDIVIDUALS WITH CHRONIC LYMPHATIC LEUCEMIA

| SUBJECT AND DATE | LEUCOCYTE COUNT PER CU.MM. (CAPILLARY BLOOD) | DIFFERENTIAL COUNT | | | LEUCOCYTE COUNT PER CU.MM. (SUSPENSION) | ALKALINE PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ALKALINE PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD | ACID PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | BLASTS | MATURE GRANULOCYTES | LYMPHOCYTES | | MG. PHOSPHORUS LIBERATED PER HOUR | | | | |
| W. Bl. | | | | | | | | | | |
| 8/16/50 | 213,750 | 0 (0) | 8,550 (4.0) | 205,200 (96.0) | 577,500 | 2.5 | 0.054 | 1.1 | 0.024 | Preirradiation |
| 9/5/50 | 3,230 | 0 (0) | 695 (21.5) | 2,535 (78.5) | 6,233 | 3.8 | 0.001 | 105.6 | 0.034 | Postirradiation |
| H. G. | | | | | | | | | | |
| 8/18/50 | 47,850 | 0 (0) | 4,785 (10.0) | 43,065 (90.0) | 56,700 | 53.8 | 0.257 | 13.0 | 0.062 | Bronchopneumonia |
| 9/25/50 | 38,500 | 0 (0) | 3,272 (8.5) | 35,228 (91.5) | 82,125 | 10.4 | 0.040 | 4.3 | 0.017 | Clinically free of infection |
| 1/8/51 | 6,288 | 0 (0) | 3,018 (48.0) | 3,270 (52.0) | 15,163 | 57.3 | 0.036 | 28.9 | 0.018 | Recurrent bronchopneumonia |
| G. Mc. | | | | | | | | | | |
| 8/29/50 | 58,600 | 0 (0) | 293 (0.5) | 58,307 (99.5) | 150,893 | 3.8 | 0.022 | 2.6 | 0.016 | Receiving ACTH |
| 10/2/50 | 22,613 | 0 (0) | 452 (2.0) | 22,161 (98.0) | 78,250 | 3.5 | 0.008 | 1.6 | 0.003 | ACTH discontinued |
| E. G. | 15,750 | 0 (0) | 2,284 (14.5) | 13,466 (85.5) | 30,900 | 19.4 | 0.031 | 7.1 | 0.012 | 30 days postirradiation |
| G. B. | 7,838 | 0 (0) | 6,114 (78.0) | 1,724 (22.0) | 17,148 | 26.5 | 0.021 | 31.4 | 0.025 | Postirradiation |
| W. H. | 1,675 | 0 (0) | 134 (8.0) | 1,541 (92.0) | 3,275 | 3.7 | 0.001 | 18.2 | 0.003 | Postirradiation |
| K. G. | 2,238 | 0 (0) | 1,141 (51.0) | 1,097 (49.0) | 11,425 | 9.8 | 0.002 | 38.0 | 0.009 | Postirradiation |
| S. O. | 89,000 | 0 (0) | 5,340 (6.0) | 83,660 (94.0) | 51,175 | 3.6 | 0.032 | 4.6 | 0.041 | |
| E. Z. | 24,700 | 0 (0) | 7,410 (30.0) | 17,290 (70.0) | 43,650 | 10.3 | 0.026 | 8.4 | 0.021 | |
| L. L. K. | 12,088 | 121 (1.0) | 2,780 (23.0) | 9,180 (76.0) | 27,500 | 12.8 | 0.015 | 8.7 | 0.011 | |
| C. H. | 9,663 | 0 (0) | 2,512 (26.0) | 7,151 (74.0) | 24,088 | 68.2 | 0.066 | 16.9 | 0.016 | Receiving ACTH |
| T. D. | 19,500 | 195 (1.0) | 780 (4.0) | 18,525 (95.0) | 26,525 | 2.9 | 0.006 | 2.7 | 0.005 | |
| Mean | | | | | | 20.8 | 0.039 | 18.3 | 0.020 | |

TABLE II. PHOSPHATASE ACTIVITY IN INDIVIDUALS WITH ACUTE LEUCEMIA

| SUBJECT AND DATE | LEUCOCYTE COUNT PER CU.MM. (CAPILLARY BLOOD) | DIFFERENTIAL COUNT | | | LEUCOCYTE COUNT PER CU.MM. (SUSPENSION) | ALKALINE PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ALKALINE PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD | ACID PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | BLASTS | MATURE GRANULOCYTES | LYMPHOCYTES | | MG. PHOSPHORUS LIBERATED PER HOUR | | | | |
| E. Y. | 10,275 | 3,596 (35.0) | 0 (0) | 6,679 (65.0) | 28,550 | 0.0 | 0.000 | 7.4 | 0.008 | |
| A. G. | 179,500 | 156,165 (87.0) | 7,180 (4.0) | 16,155 (9.0) | 162,500 | 0.0 | 0.000 | 3.5 | 0.062 | |
| C. R. | 6,225 | 3,115 (50.0) | 1,740 (28.0) | 1,370 (22.0) | 19,175 | 6.2 | 0.004 | 14.4 | 0.009 | |
| E. Wa. | 4,363 | 1,527 (35.0) | 219 (3.0) | 2,705 (62.0) | 14,525 | 2.8 | 0.001 | 6.3 | 0.003 | |
| H. N. | 121,500 | 106,920 (88.0) | 3,645 (3.0) | 10,935 (9.0) | 106,125 | 0.0 | 0.000 | 2.9 | 0.036 | |
| C. P. | | | | | | | | | | |
| 9/13/50 | 177,000 | 122,130 (69.0) | 1,770 (1.0) | 30,530 (30.0) | 208,225 | 1.4 | 0.026 | 3.8 | 0.068 | Untreated |
| 10/5/50 | 558,750 | 502,875 (90.0) | 5,588 (1.0) | 50,288 (9.0) | 535,400 | 1.5 | 0.084 | 2.9 | 0.163 | Untreated |
| 10/24/50 | 23,238 | 16,499 (71.0) | 232 (1.0) | 6,506 (28.0) | 24,113 | 0.3 | 0.001 | 0.0 | 0.000 | Receiving aminopterin |
| S. G. | 80,550 | 74,912 (93.0) | 806 (1.0) | 4,833 (6.0) | 98,400 | 1.7 | 0.013 | 45.7 | 0.368 | Receiving ACTH |
| E. W. | | | | | | | | | | |
| 11/22/50 | 293,500 | 149,685 (51.0) | 8,805 (3.0) | 135,010 (46.0) | 225,417 | 1.8 | 0.054 | 4.8 | 0.141 | Untreated |
| 11/29/50 | 199,025 | 139,318 (70.0) | 995 (0.5) | 58,712 (29.5) | 245,875 | 1.8 | 0.036 | 6.3 | 0.126 | Receiving ACTH since 11/25/50 |
| 12/8/50 | 9,425 | 0 (0) | 2,356 (25.0) | 7,069 (75.0) | 21,463 | 31.0 | 0.029 | 18.6 | 0.018 | Still on ACTH |
| 12/18/50 | 4,050 | 0 (0) | 2,673 (66.0) | 1,377 (34.0) | 11,313 | 48.3 | 0.020 | 46.5 | 0.019 | Still on ACTH |
| 1/15/51 | 3,600 | 0 (0) | 2,484 (69.0) | 1,116 (31.0) | 7,663 | 40.6 | 0.015 | 25.0 | 0.009 | Still on ACTH |
| Mean | | | | | | 1.6 | 0.020 | 8.9 | 0.089 | |

TABLE III. PHOSPHATASE ACTIVITY IN A MISCELLANEOUS GROUP OF PATIENTS WITH HEMATOLOGIC DISORDERS: FIGURES OUTSIDE NORMAL LIMITS ARE INDICATED WITH AN ASTERISK

| SUBJECT | LEUCOCYTE COUNT PER CU.MM. (PERIPHERAL BLOOD) | DIFFERENTIAL COUNT | | | LEUCOCYTE COUNT PER CU.MM. (SUSPENSION) | ALKALINE PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ALK. PHOSPHATASE PER W.B.C. OF 1 ML. OF BLOOD | ACID PHOSPHATASE PER $10^{10}$ LEUCOCYTES | ACID PHOSPHATASE PER LEUCOCYTES OF 1 ML. OF BLOOD | CLINICAL STATUS |
|---|---|---|---|---|---|---|---|---|---|---|
| | | GRANULOCYTES | LYMPHOCYTES | MONOCYTES | | MG. PHOSPHORUS LIBERATED PER HOUR | | | | |
| L. L. | 60,000 | 58,500 (97.5) | 900 (1.5) | 300 (0.5) | 53,300 | 124.7* | 0.750 | 27.1 | 0.160 | Polycythemia vera |
| T. M. | 22,542 | 19,161 (85.0) | 2,480 (11.0) | 902 (4.0) | 38,175 | 54.5* | 0.123 | 24.2 | 0.055 | Polycythemia vera |
| J. S. | 16,725 | 14,551 (87.0) | 1,840 (11.0) | 167 (1.0) | 25,500 | 66.9* | 0.112 | 41.3 | 0.069 | Polycythemia vera |
| R. A. | 17,900 | 15,215 (85.0) | 2,327 (13.0) | 358 (2.0) | 64,500 | 44.4 | 0.080 | 18.7 | 0.033 | Eosinophilia (11%) and cancer of tongue |
| E. R. | 11,488 | 8,587 (61.5) | 3,906 (34.0) | 517 (4.5) | 14,463 | 18.2 | 0.021 | 26.2 | 0.030 | Eosinophilia (10%) otherwise normal |
| B. D. | 48,938 | 41,108 (84.0) | 7,830 (16.0) | 0 (0) | 59,150 | 40.3 | 0.197 | 34.3 | 0.167 | Leucemoid reaction; gastric carcinoma |
| H. E. | 5,025 | 4,020 (80.0) | 854 (17.0) | 0 (0) | 7,894 | 46.5 | 0.023 | 13.6 | 0.007 | Multiple myeloma |
| J. McG. | 8,063 | 7,418 (92.0) | 645 (8.0) | 0 (0) | 19,825 | 49.5 | 0.040 | 23.9 | 0.019 | Multiple myeloma (receiving cortisone) |
| D. L. | 6,450 | 4,902 (76.0) | 1,484 (23.0) | 65 (1.0) | 13,475 | 12.7 | 0.008 | 28.7 | 0.019 | Lymphosarcoma |
| R. H. | 15,113 | 6,045 (40.0) | 8,917 (59.0) | 151 (1.0) | 19,538 | 27.4 | 0.041 | 21.4 | 0.032 | Lymphosarcoma |
| W. W. | 19,600 | 8,624 (44.0) | 10,976 (56.0) | 0 (0) | 44,250 | 20.6 | 0.040 | 11.2 | 0.022 | Lymphosarcoma |
| G. H. | 7,313 | 5,119 (70.0) | 2,048 (28.0) | 146 (2.0) | 18,513 | 14.2 | 0.010 | 24.8 | 0.018 | Leucemia cutis |
| F. B. | 4,375 | 2,840 (65.0) | 918 (21.0) | 568 (13.0) | 9,638 | 2.5* | 0.001 | 31.9 | 0.014 | Paroxysmal nocturnal hemoglobinuria |
| C. M. | 6,575 | 4,405 (67.0) | 1,973 (30.0) | 197 (3.0) | 10,608 | 20.7 | 0.014 | 40.2 | .027 | Hodgkin's disease |
| J. J. | 8,950 | 7,966 (89.0) | 984 (11.0) | 0 (0) | 18,608 | 73.3* | 0.066 | 34.9 | 0.031 | Agnogenic myeloid metaplasia |

activity, so expressed, ranged from 13.7 to 36.8 with a mean of 21.9. Alkaline phosphatase expressed in terms of milligrams of phosphorus liberated per leucocytes of 1 ml. of blood ranged in the normal group from 0.007 to 0.035 with a mean of 0.018. The corresponding values for the acid phosphatase ranged from 0.009 to 0.035 with a mean of 0.015.

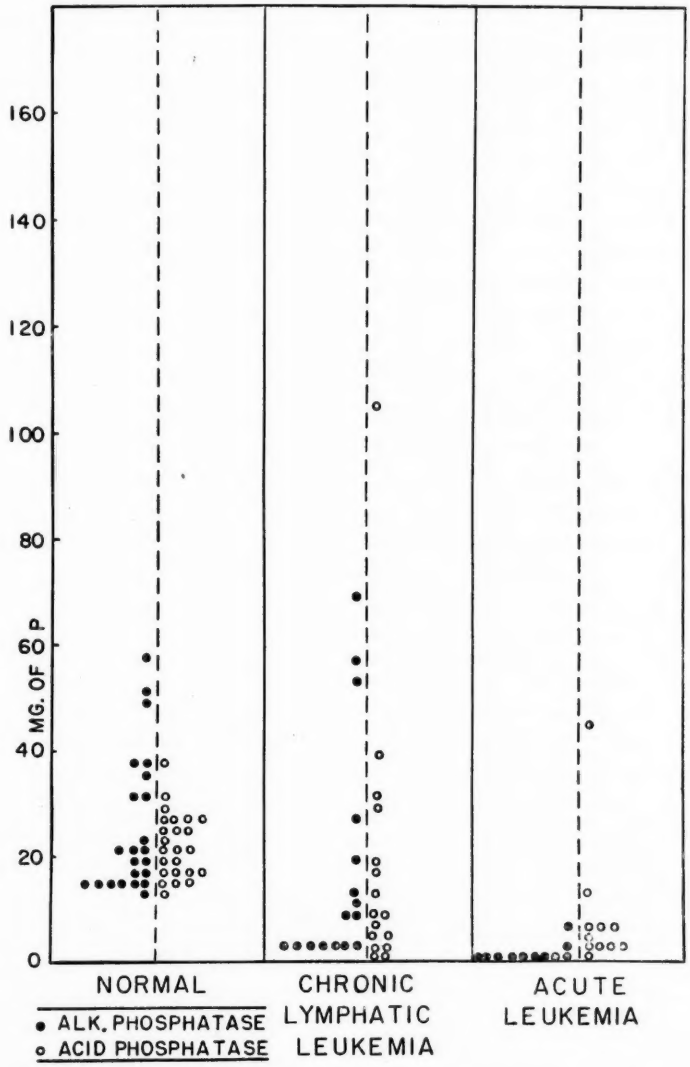


Fig. 1.—Phosphatase activity of leucocytes of normal individuals and subjects with chronic lymphatic and acute leucemia. Phosphatase activity is here expressed in terms of milligrams of phosphorus liberated per hour by $10^{10}$ leucocytes in saline suspension. *Black dots* refer to alkaline phosphatase and *circles* indicate acid phosphatase.

*Chronic Lymphatic Leucemia (See Table I).*—Sixteen determinations were done on twelve individuals in various stages of chronic lymphatic leucemia. A wide range of leucocyte counts in peripheral blood is represented, the spread varying from 1,675 to 213,750 cells per cubic millimeter. The patients whose

leucocyte counts were below 10,000 (with the exception of Patient H. G.) were established cases of chronic lymphatic leucemia who had recently received radiation or ACTH therapy. The range of alkaline phosphatase levels per $10^{10}$ leucocytes was 2.5 to 68.2 with a mean of 20.8. Only three determinations were higher than 26.5. Two of these were found in Patient H. G. (53.8 and 57.3) who was on both occasions suffering from bronchopneumonia. Between these determinations, he was free of clinical evidence of pneumonia and was found to have an alkaline phosphatase level of 10.4. This sequence of events may represent an example of the increasing alkaline phosphatase activity which accompanies infection, a phenomenon which occurs so strikingly in physiologic leucocytosis of infection, unassociated with leucemia. If it is true that the alkaline phosphatase is reposited chiefly in the cells of the granulocytic series, it is interesting to note that between the first and second determinations (Table I) Patient H. G. showed essentially no hematologic change, the percentages of myeloid cells remaining about the same (10.0 and 8.5 per cent). Despite this, a marked drop in alkaline phosphatase activity accompanied recovery from infection. However, between the second and third determinations the peripheral blood had changed toward normal, the count being 6,288 per cubic millimeter with 48.0 per cent granulocytes. The return to a higher level of alkaline phosphatase may here represent the higher total contribution of a greater number of granulocytes per $10^{10}$ cells, a total probably made even higher by the presence of infection. If this does, indeed, represent an example of elevation of leucocyte alkaline phosphatase due to infection, it may be said that this individual has responded in a more nearly normal manner to the stimulus of infection than did a corresponding patient with chronic myelocytic leucemia whose preinfection level of 1.2 rose to only 14.4 during severe infection.

The third instance of a higher level of alkaline phosphatase activity was in Patient C. H. (68.2) who at the time was receiving ACTH therapy. Patient G. Mc., whose alkaline phosphatase was 3.8, was also receiving ACTH so it cannot be concluded that the higher level was due to the hormone. In addition, Patient C. H. had 26.0 per cent myeloid cells compared with the 0.5 per cent of patient G. Mc. This may in part account for C. H.'s higher level of alkaline phosphatase activity per $10^{10}$ leucocytes.

Interestingly, one patient (W. Bl.) showed an essentially unchanged level of alkaline phosphatase activity per $10^{10}$ cells despite a marked drop in the number of leucocytes per cubic millimeter of peripheral blood induced by radiation therapy. The preirradiation count was 213,750 with 4.0 per cent myeloid cells and an alkaline phosphatase of 2.5. The postirradiation count was 3,230 with 21.5 per cent myeloid cells, and an alkaline phosphatase of 3.8. Here, the increased percentage of myeloid cells apparently made little difference in the alkaline phosphatase activity of the theoretical aliquot of $10^{10}$ cells. The alkaline phosphatase accounted for by the leucocytes of 1 ml. of blood dropped with therapy, from 0.054 to 0.001 (a fifty-four fold drop) as the absolute number of circulating granulocytes per cubic millimeter dropped from 8,550 to 695 (a twelvefold drop). Apparently, each post-treatment granulocyte contained less alkaline phosphatase. Those patients with the lowest alkaline phosphatase levels (W. Bl., 2.5; G. Mc., 3.8; S. O., 3.6; W. H., 3.7; and T. D., 2.9) were individ-

uals whose lymphocyte percentages were decidedly in the higher ranges (W. Bl., 96.0 per cent; G. Mc., 99.5 per cent; S. O., 94.0 per cent; W. H., 92.0 per cent; T. D., 96.0 per cent.

The acid phosphatase levels per $10^{10}$ cells ranged from 1.10 to 105.6 with a mean of 18.3. With the exception of the figure of 105.6 recorded in Patient W. Bl. post irradiation, the over-all trend in acid phosphatase values appeared to be toward lower than normal values. The mean, excluding the value of 105.6, was 12.5, a figure distinctly lower than the normal mean of 21.9. This one exceptional value is interesting in view of the proportional change which took place in the peripheral white blood count. The count dropped as a result of radiation from 213,750 to 3,230, the ratio between the pre- and post-therapy count being 66:1, as the acid phosphatase increased from 1.1 to 105.6, a ninety-six fold increase. As a result of this reciprocal change, the amount of acid phosphatase accounted for by the leucocytes of 1 ml. of blood remained essentially the same (0.024 and 0.034). No other cases have been similarly followed, consequently the significance of this observation cannot be evaluated at present.

The alkaline phosphatase values accounted for by the leucocytes of 1 ml. of blood ranged from 0.001 to 0.257 with a mean of 0.039. The corresponding values for acid phosphatase activity ranged from 0.003 to 0.062 with a mean of 0.020.

*Acute Leucemia* (*Table II*).—In this group, fourteen determinations were performed on eight patients. One patient (E. W.) was followed repeatedly through a course of ACTH therapy and will be discussed below. In all cases, the percentage of blast forms was 35 per cent or higher. In six of the eight patients the percentage of blasts exceeded 50 per cent. The range of alkaline phosphatase activity per $10^{10}$ leucocytes (excluding the post-treatment values of Patient E. W.) was 0.0 to 6.2, the mean being 1.6. Acid phosphatase ranged from 0 to 45.7, averaging 8.9. Both means are considerably lower than normal. The amount of alkaline enzyme activity per leucocytes of 1 ml. of blood was 0.000 to 0.084, averaging 0.020. The acid phosphatase activity per leucocytes of 1 ml. of blood ranged from 0.000 to 0.368 with a mean of 0.089. The tendency toward low levels for both alkaline and acid phosphatase activity per $10^{10}$ cells suggests that the blast form is poor in phosphatase, the activity being attributable chiefly to the more mature cells. This inference is supported by histochemical data.[4]

Patient E. W., a young man with recently diagnosed acute leucemia, showed a striking clinical and hematologic response to ACTH, returning quickly to an outwardly normal state with a normal leucocyte count and differential picture. ACTH (100 mg. per day) was continued for a prolonged period, during which the blood picture, except for a slight leucopenia, was indistinguishable from normal. Alkaline phosphatase per $10^{10}$ leucocytes, originally 1.8, rose to 31.0 and then 48.3 and 40.6, figures within the normal range. Similarly, the acid phosphatase which had been 4.8 rose to a normal level and remained there. This patient is in striking contrast to the several recorded cases of myelocytic and lymphatic leucemia in which a return to normal peripheral blood leucocyte levels, usually induced by therapy other than ACTH, was unaccompanied by a return to normal phosphatase levels. This series of results suggests that in this

individual the trend toward normal in the rate of proliferation and morphology of the leucocytes was accompanied by similar change in the levels of phosphatase activity. Undoubtedly, the total activity of an aliquot of $10^{10}$ cells was brought nearer to normal by the therapeutically induced disappearance of phosphatase-poor blast forms, leaving behind a complement of morphologically normal cells with normal levels of activity.

*Miscellaneous Hematologic Disorders* (*Table III*).—This group consisted of fifteen individuals with an assortment of hematologic disorders including polycythemia vera (three cases), eosinophilia (two cases), lymphosarcoma (three cases), multiple myeloma (two cases), Hodgkin's disease, leucemoid reaction, agnogenic myeloid metaplasia, leucemia cutis, and paroxysmal nocturnal hemoglobinuria. The results which are outside the normal range are denoted in Table III by asterisks. Several points here deserve emphasis. It is noteworthy that all three polycythemic patients showed elevations of alkaline phosphatase activity per $10^{10}$ cells, the mean of the three being 82.0 as compared with the mean of the normal group (25.8) and that of the group with chronic myelocytic leucemia (4.0). Each of these individuals had elevated peripheral blood counts with a shift toward immaturity in the differential formula. The remainder of the determinations recorded in the miscellaneous group are within normal limits with two exceptions. Patient J. J., an elderly man with massive splenomegaly and a clinical picture suggesting agnogenic myeloid metaplasia, had an alkaline phosphatase level per $10^{10}$ cells of 73.3, a figure which is far removed from the means of the leucemic groups so far tested. Patient F. B., a young man with a severe chronic paroxysmal hemoglobinuria and persistent moderate leucopenia, had an alkaline phosphatase level of 2.5, a figure considerably below normal. In this entire group the acid phosphatase values per $10^{10}$ cells were within the normal range.

## DISCUSSION

The description of patterns of phosphatase activity has been extended to include chronic lymphatic leucemia, acute leucemia, and a variety of miscellaneous hematologic conditions including polycythemia vera and agnogenic myeloid metaplasia. Whereas the cells of physiologic leucocytosis are characterized by a high alkaline-normal acid phosphatase content and chronic myelocytic leucemia by a low alkaline-normal to high acid phosphatase pattern, those of chronic lymphatic leucemia and acute blastic leucemia tend to show low levels of activity in both alkaline and acid phosphatase. The low alkaline phosphatase activity in these groups may be attributable to the low percentage of myeloid cells, the chief repository of alkaline phosphatase. Thus in several instances in which the differential formulas have been brought nearer normal by therapy, the alkaline phosphatase per $10^{10}$ leucocytes has also tended to approach normal levels. One patient with lymphatic leucemia (H. G.) apparently developed a pronounced increase in leucocyte alkaline phosphatase in response to infection, the magnitude of response being much greater than in a corresponding patient with myelocytic leucemia. Three cases of polycythemia vera are recorded with unit cell alkaline phosphatase levels that are decidedly above normal. This observation, in the light of Wagner's data[5] showing elevated glycogen levels in the leucocytes of

polycythemia vera, raises interesting questions concerning the nature of the metabolic changes in polycythemia. In addition, the single case of agnogenic myeloid metaplasia was found to have a distinctly elevated alkaline phosphatase level per $10^{10}$ cells. In a recent editorial, Dameshek[6] has stressed the unsatisfactory state of our present understanding of the so-called "myeloproliferative syndromes." He states that "polycythemia vera, undoubtedly a panmyelopathy, has varients which cannot be distinguished from chronic granulocytic leukemia or myeloid metaplasia." Possibly, the accumulation of more data of this type will provide criteria of a different type for the differentiation of the various "transition forms" and subgroups. It would appear on the basis of the present data that the leucocytes of polycythemia vera and myeloid metaplasia are distinguishable from those of leucemia by their phosphatase content.

Leucocyte histamine levels done in this laboratory have shown abnormally low values in both lymphatic and acute leucemia presumably due to the fact that histamine resides chiefly in the cells of the myeloid series. Thus, more data have been recorded to add to the growing awareness of the chemical differences between the leucocytes of health and disease. It is hoped that further studies of this type will delineate more completely the wide biochemical differences between normal and abnormal leucocytes and provide eventually a clue to the basic nature of the processes of leucocytosis and leucemia.

## SUMMARY

Determinations of alkaline and acid phosphatase activity have been done on the leucocytes of chronic lymphatic leucemia, acute leucemia, and a variety of hematologic disorders. Both chronic lymphatic and acute leucemia tend to show low acid and alkaline activity levels per $10^{10}$ cells as compared with chronic myelocytic leucemia in which the alkaline phosphatase is low and acid phosphatase activity greater than normal and with physiologic leucocytosis in which the alkaline phosphatase is extremely high. The low phosphatase activity in these groups may be attributable to the low percentages of normal myeloid and lymphoid cells per $10^{10}$ leucocytes. Three cases of polycythemia vera and one case of agnogenic myeloid metaplasia showed elevated leucocyte alkaline phosphatase activity. These changes may be clues to fundamental metabolic aspects of these conditions.

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# OBSERVATIONS ON THE CHARACTERISTICS OF AN UNUSUAL CIRCULATING ANTICOAGULANT

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WITHIN the last few years, a number of patients have been described whose plasmas have exhibited anticoagulant activity against normal blood or plasma. Usually, attention was called to these patients by the observation of a prolonged clotting time. In contradistinction to patients with uncomplicated hemophilia, the clotting time of normal blood was prolonged by the addition of small amounts of the patient's blood when tested in vitro.

In a few cases, the anticoagulant appeared to inhibit thrombin.[1] In others, some substance in plasma was thought to interfere with the activation of the thromboplastic factor of plasma from its precursor. In still others, the inhibitor appeared to delay the conversion of prothrombin to thrombin by interfering with the action of thromboplastin. The reported cases have been reviewed by Dreskin and Rosenthal.[2]

This report describes an unusual anticoagulant present in the plasma of a patient with subacute bacterial endocarditis. At 25° C. the clotting time of the patient's blood was greatly prolonged, but at 37° C. it was usually within normal limits. Small amounts of the patient's whole blood delayed the clotting time of normal blood at 25° C. but not at 37° C. In the absence of the formed elements of the blood, however, the circulating anticoagulant was active at both temperatures. Subsequent investigation indicated that the substance probably delayed the conversion of prothrombin to thrombin, an activity opposed by the presence of blood cells or by excesses of thromboplastin.

## CASE REPORT

R. D., a 40-year-old white welder of Italian descent, entered the University Hospitals on Dec. 17, 1950, with a story of gradually increasing weakness for six months. Four months prior to admission he noted painful, hot, swellings of the right wrist and both elbows which persisted for three days. Two weeks before admission he developed gross hematuria which lasted four days, followed by cloudy urine and frequency of urination for the ten days prior to admission. Submucosal hemorrhages of the bladder were seen upon cystoscopic examination in another hospital.

On admission, the temperature, pulse, and respirations were within normal limits. The patient was well nourished. His face was flushed and there was mild periorbital edema. Periarteriolar retinal hemorrhages were observed. The heart and lungs were not remarkable; the blood pressure was 175/105 mm. Hg. The liver was palpable 3 cm. below the costal margin, but the spleen was not palpable. A splinter hemorrhage was present beneath the nail of the fourth left finger. There was pronounced tenderness and pain on motion of the left wrist and elbow without evident swelling or increased heat. Deep tendon reflexes were equal and normally active, but a positive Babinski sign was elicited on the left.

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Urinalysis revealed gross hematuria with rare granular and red cell casts, 1+ albuminuria, and normal urinary specific gravity. Hemoglobin, 14 Gm. per 100 ml.; erythrocytes, 4.31 million per cubic millimeter; hematocrit, 38 per cent; leucocytes, 9,050 per cubic millimeter. Differential cell count was normal. The platelet count was 164,000 per cubic millimeter (normal 250,000 to 500,000), and good clot retraction occurred within four hours. The bleeding time (Duke) was 11 minutes, the clotting time (Lee-White) was 73 minutes at room temperature (about 25° C.), and a tourniquet test with the cuff at 140 mm. Hg for 5 minutes was strongly positive. The plasma fibrinogen concentration was 800 mg. per 100 milliliters. The cephalin flocculation test was 4+, but the thymol turbidity, total protein, serum bilirubin, and bromsulfalein retention tests were within normal limits. Maximal urea clearance was 67 per cent and 41 per cent of normal, and the blood urea nitrogen was 13 mg. per 100 milliliters. Examination of the spinal fluid revealed an initial pressure of 210 mm. of water, 3 mononuclear cells per cubic millimeter, protein 55 mg. per 100 ml., sugar 55 mg. per 100 ml., and chloride 122 meq. per liter. Three blood cultures taken early during hospitalization were negative. Electrophoretic analysis of serum revealed a decrease in the albumin fraction and an increase in the gamma, theta, and alpha-1 and alpha-2 fractions of the globulin. It was noted that flocculation developed in the patient's serum when it was allowed to stand in the refrigerator overnight. This was interpreted as evidence for the existence of cryoglobulin; 4.7 mg. per 100 ml. of this material was present in the serum. In addition, porphobilinogen was found in the urine from time to time and was present in greatest quantity just prior to death.

The patient's condition became progressively worse with development of complete paralysis of the left arm and partial paralysis of the left leg. On Jan. 7, 1951, the first of several grand mal seizures occurred. On the following day he developed abdominal pain associated with rigidity of the abdominal muscles and leucocytosis. An exploratory laparotomy was performed, but no abnormality was found. The patient tolerated this procedure without abnormal bleeding. During the final three weeks he was moderately febrile. Preterminally his fever rose to 40° C. Penicillin and streptomycin were given during the last two days of life. The patient died from circulatory collapse on the twenty-seventh hospital day.

At necropsy, a partially organized bacterial endocarditis was present on an otherwise normal mitral valve. There were numerous septic infarcts in the brain, liver, spleen, and kidneys. Both adrenal glands contained large hemorrhages in the medulla, but the cortices were preserved. All cultures were negative in spite of the finding of bacteria in the histologic sections of the heart valves. The final pathologic diagnosis was subacute bacterial endocarditis, bacterial agent unidentified.

## METHODS

In this paper, the term *thromboplastin* refers to a substance initiating the conversion of prothrombin to thrombin in the presence of calcium. *Thromboplastinogen* designates the substance or substances in plasma which, when activated, may initiate this conversion. Its active form has been designated "plasma thromboplastic factor."

*Oxalated blood* was prepared by mixing 9 parts of whole blood with 1 part of 0.1M sodium oxalate solution. *Oxalated plasma* was separated by centrifugation at 2,500 r.p.m. at room temperature in a Servall Model G-2 angle centrifuge (rim diameter 15 inches).

*Platelet-deficient plasma* was obtained by the technique described by Jacques[3] and by Conley and associates.[4] Blood was drawn without anticoagulant using silicone-coated equipment. The formed elements, including the platelets, were removed by centrifugation at 10,500 r.p.m. for ten minutes in a refrigerated Servall Superspeed I angle centrifuge (rim diameter 9 inches). The platelet count on such plasma was uniformly less than 50 per cubic millimeter.

The *buffer* solution used in these experiments was composed of 7.3 Gm. sodium chloride, 2.76 Gm. barbital, and 2.06 Gm. sodium barbital diluted to a volume of 1 liter with distilled water, with a resulting pH of 7.5.

*Thromboplastin* was prepared from acetone-extracted rabbit brain from which the large blood vessels had been removed. The brains were dried in a desiccator, pulverized, and suspended in 0.9 per cent saline in a concentration of 250 mg. per 4.9 ml. of saline. To this

was added 0.1 ml. of 0.1M sodium oxalate. This suspension was heated at 54° C. for fifteen minutes, and centrifuged at 1,800 r.p.m. for twenty seconds. The supernatant was frozen until used.

*Washed blood cells* were prepared in the following manner. Normal blood cells were separated from oxalated whole blood by centrifugation at 2,500 r.p.m. at room temperature. The cells were then washed three times with buffer, and diluted to twice the volume of the cells. Care was taken to prevent loss of leucocytes in the washing process.

*Prothrombin-deficient plasma* was prepared by incubating oxalated plasma at 37° C. for ten minutes with about one-fifth its volume of powdered barium sulfate. The supernatant plasma was then separated by centrifugation. Such prothrombin-deficient plasma failed to clot within twenty-four hours after addition of equal parts of thromboplastin and 0.025M calcium chloride solution.

*Cryoglobulin-deficient plasma* was prepared by refrigerating the patient's oxalated plasma at 4° C. for forty-eight hours. The cryoglobulins were then removed by centrifugation at room temperature.

*Clotting times* were performed in Pyrex glass tubes with an internal diameter of 11 millimeters. The effects of thrombin and thromboplastin were tested (usually in duplicate or triplicate) in Pyrex tubes with an internal diameter of 8 millimeters. The averages of these determinations are recorded in the tables.

Dialyzed plasma was prepared by dialyzing oxalated platelet-deficient plasma in Visking No-Jax cellophane casings (diameter, 1 in.) against frequent changes of barbital buffer at 4° C.

## RESULTS

*Anticoagulant Effect of Patient's Whole Blood and Plasma.*—The effect of varying amounts of the patient's whole blood on the clotting time of normal whole blood was tested at 25° C. and 37° C. (Table I). In two experiments the clotting time of normal blood at 25° C. was prolonged by the addition of the patient's blood. However, at 37° C. no significant prolongation occurred.

In contradistinction, the patient's oxalated plasma prolonged the recalcified clotting time of normal oxalated plasma at both 25° C. and 37° C. (Table II).

These results indicate that the patient's plasma contained a substance or substances which inhibited the coagulation of normal blood. However, the inhibitory effect could not be demonstrated with whole blood at 37° C.

*Effect of Thrombin on the Clotting Time of the Patient's Oxalated Plasma.*—Varying amounts of thrombin* were added to the patient's oxalated plasma at 25° C. Decreasing the amount of thrombin caused similar prolongation of the clotting times of both the patient's and normal control oxalated plasmas. (Table III.) In a second experiment, similar effects were noted at 25° C. and 37° C.

TABLE I. THE ANTICOAGULANT EFFECT OF PATIENT'S WHOLE BLOOD ON NORMAL WHOLE BLOOD AT 25° C. AND 37° C.

| Tubes | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Patient blood (ml.) | 2 | 1.6 | 1.2 | 0.8 | 0.4 | 0 |
| Control blood (ml.) | 0 | 0.4 | 0.8 | 1.2 | 1.6 | 2 |
| Clotting time (min.) 25° C. | 78 | 43½ | 46 | 27¼ | 20 | 9¼ |
| Clotting time (min.) 37° C. | 13¼ | 10 | 8½ | 10 | 8¼ | 9½ |

*Thrombin was obtained through the courtesy of Parke, Davis & Co., Detroit, Mich.

TABLE II. THE ANTICOAGULANT EFFECT OF THE PATIENT'S OXALATED PLASMA ON THE RECALCIFIED CLOTTING TIME OF NORMAL OXALATED PLASMA AT 25° C. AND 37° C.

| Tubes | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Patient plasma (ml.) | 0 | 0.1 | 0.2 | 0.3 | 0.4 |
| Control plasma (ml.) | 0.4 | 0.3 | 0.2 | 0.1 | 0 |
| $CaCl_2$ 0.025M (ml.) | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Clotting time (min.) 25° C. | 6 | 19¾ | 14½ | 21 | 45 |
| Clotting time (min.) 37° C. | 2½ | 4¼ | 5½ | 8 | 15 |

TABLE III. EFFECT OF VARYING AMOUNTS OF THROMBIN ON THE CLOTTING TIME OF OXALATED PLASMA

Clotting time (sec.) at 25° C. of 0.2 ml. oxalated plasma on adding 0.2 ml. thrombin solution

| SOURCE OF PLASMA | INITIAL THROMBIN CONCENTRATION (NIH UNITS/ML.) 10 | 5 | 2.5 | 1.25 |
|---|---|---|---|---|
| Patient | 12 | 18 | 30 | 57 |
| Normal control | 17.5 | 23 | 34 | 65 |

These data demonstrate that the patient's plasma did not inhibit the conversion of fibrinogen to fibrin by thrombin.

*Studies of the Rate of Conversion of Prothrombin to Thrombin.*—A prothrombin consumption test was performed using the technique of Stefanini and Crosby.[5] Blood was allowed to clot at 25° C. and 37° C.; the clotting time at 25° C. was 130 minutes and at 37° C. was 12 minutes. One hour after the clot was complete the serum was removed and the prothrombin time measured (Table IV). The results indicate that at 25° C. the residual prothrombin time was significantly shorter in the patient's serum than in normal serum. At 37° C., however, there was little difference between the two bloods.

Oxalated plasma was separated by centrifugation at 1,000 r.p.m. and at 3,000 r.p.m. in an International I Type SB centrifuge for five minutes and the recalcified clotting times of the two samples were compared (Table V). Both at 25° C. and 37° C., the clotting time of the plasma which had been centrifuged at 3,000 r.p.m. was significantly longer than that of the plasma centrifuged at 1,000 r.p.m.

The results of both of these tests suggest that a defect may have been present in the conversion of prothrombin to thrombin. The defect was not demonstrated at 37° C. by the prothrombin consumption test performed with whole blood. However, it was demonstrated at this temperature by the differential centrifugation test of Quick[6] in which oxalated plasma was tested.

*The Effect of the Anticoagulant on Prothrombin.*—The impaired conversion of prothrombin to thrombin might have been the result of inhibition of pro-

TABLE IV. RESIDUAL PROTHROMBIN TIME OF SERUM OBTAINED FROM WHOLE BLOOD WHICH WAS ALLOWED TO CLOT AT 25° C. AND 37°C.

| SERUM | RESIDUAL PROTHROMBIN TIME (SEC.) 25° C. | 37° C. |
|---|---|---|
| Patient | 13 | 41 |
| Control | 94 | 26 |

2.5 ml. portions of whole blood were allowed to clot at 25° C. and at 37° C. One hour after clotting was complete, the serum was separated by centrifugation. One tenth milliliter of 0.1M sodium citrate was then added to 0.9 ml. of serum and the mixture incubated at 37° C. for 30 minutes. The prothrombin content of the serum was then estimated at 37° C. by mixing 0.1 ml. of serum, 0.1 ml. of normal prothrombin-deficient plasma, 0.1 ml. of thromboplastin, and 0.1 ml. of 0.025M $CaCl_2$.

TABLE V. THE RECALCIFIED CLOTTING TIME OF OXALATED PLASMA SUBJECTED TO DIFFERENTIAL CENTRIFUGATION

Clotting time (min.) of 0.1 ml. of oxalated plasma on adding 0.1 ml. of 0.025M $CaCl_2$

| SOURCE OF PLASMA | TEMP. | 1,000 R.P.M. FOR 5 MIN. | 3,000 R.P.M. FOR 5 MIN. |
|---|---|---|---|
| Patient | 25° C. | 21 | 31 |
| Control | 25° C. | 4 | 5 |
| Patient | 37° C. | 3¾ | 8¼ |
| Control | 37° C. | 2 | 2½ |

thrombin, accelerator globulin, thromboplastin, thromboplastinogen, or some as yet undescribed factor. If the anticoagulant inactivated prothrombin itself, one might expect that the inhibition would manifest itself more strongly when the concentration of prothrombin was decreased. To study this, the patient's plasma was serially diluted with his own prothrombin-deficient plasma, and the prothrombin time of the mixtures determined (Table VI).

The results of this experiment demonstrate that there was no essential difference in the behavior of the patient's plasma and of a control's plasma upon dilution with prothrombin-deficient plasma. As the prothrombin was diluted the prothrombin time was prolonged to a similar degree in each case. It seemed unlikely from this experiment, therefore, that the anticoagulant inactivated prothrombin itself. As the proportion of inhibitor to prothrombin increased, the patient's prothrombin was converted to thrombin equally as well as was the control's plasma. Indeed, the patient's prothrombin time was prolonged relatively less by dilution than that of a control. Furthermore, this experiment establishes the presence of a normal amount of prothrombin in the patient's plasma, a deficiency of which would complicate the interpretation of some of the tests utilizing the prothrombin time.

TABLE VI. EFFECT OF THE DILUTION OF PROTHROMBIN ON THE RATE OF CLOTTING OF OXALATED PLASMA BY THROMBOPLASTIN

Clotting time (sec.) of mixture of 0.2 ml. portions of dilutions of oxalated plasma,* 0.2 ml. of rabbit brain thromboplastin, and 0.2 ml. of 0.025M $CaCl_2$

| SOURCE OF PLASMA | TEMP. | CONCENTRATION OF PROTHROMBIN | | | |
|---|---|---|---|---|---|
| | | 100% | 70% | 40% | 10% |
| Patient | 25° C. | 36 | 37 | 37 | 52 |
| Control | 25° C. | 23 | 24 | 25 | 44 |
| Patient | 37° C. | 21 | 19 | 21 | 34 |
| Control | 37° C. | 15 | 16 | 18 | 35 |

*Each subject's oxalated plasma was diluted with his own prothrombin-deficient plasma.

TABLE VII. THE EFFECT OF VARYING AMOUNTS OF THROMBOPLASTIN ON THE PROTHROMBIN TIME OF OXALATED PLASMA

Clotting time (sec.) of mixtures of 0.2 ml. of oxalated plasma, 0.2 ml. portions of dilutions of rabbit brain thromboplastin,* and 0.2 ml. of 0.025M $CaCl_2$

| SOURCE OF PLASMA | TEMP. | DILUTION OF THROMBOPLASTIN | | | | |
|---|---|---|---|---|---|---|
| | | UNDILUTED | 1/10 | 1/50 | 1/100 | 1/200 |
| Patient | 25° C. | 25 | 49 | 104 | 121 | 171 |
| Control | 25° C. | 21 | 30 | 41 | 50 | 66 |
| Patient | 37° C. | 16 | 27 | 44 | 55 | 85 |
| Control | 37° C. | 16 | 21 | 29 | 37 | 42 |

*Rabbit brain thromboplastin was diluted serially with buffer.

*The Effect of Dilute Thromboplastin on the Prothrombin Time of the Patient's Plasma.*—If the inhibitor interfered with the action of thromboplastin on prothrombin, one would expect that the anticoagulant effect would become more pronounced as thromboplastin was diluted (Table VII). It was clear that the anticoagulant effect was, in fact, progressively greater as thromboplastin was diluted. Accordingly, one can conclude that at least one action of the circulating anticoagulant was directed in some manner against the activation of prothrombin by thromboplastin.

*Properties of the Anticoagulant.*—Allen and associates[7] have reported the presence of heparin-like anticoagulants in the blood of patients with a variety of diseases. This was demonstrated by determining the amount of protamine sulfate needed to neutralize the effect of a measured amount of heparin added to the patient's blood. The amount of protamine needed to neutralize the anticoagulant effect of 1 mg. of heparin in 11 ml. of whole blood was 0.02 mg. per milliliter greater for the patient than for a normal control, an amount probably within the limits of error of the method in this laboratory. However, the addition of protamine sulfate directly to the patient's oxalated prothrombin-deficient plasma in final concentration ranging from 0.025 to 100 mg. per milliliter did not alter the plasma's inhibitory effect on the recalcified plasma clotting time. These properties were unlike those described for heparin-like substances.

The circulating anticoagulant was observed regularly in plasma from which prothrombin had been removed by incubation with barium sulfate. This prothrombin-deficient plasma inhibited the action of a dilute thromboplastin suspension on normal oxalated plasma. The anticoagulant was nondialyzable, and was partially inactivated by heating prothrombin-deficient plasma to 56° C. for five minutes. The anticoagulant activity in oxalated platelet-deficient plasma decreased during prolonged storage at 4° C. in Pyrex tubes.

The action of the anticoagulant was demonstrable in plasma at both 25° C. and 37° C. However, in the presence of whole blood, the inhibitory effect was much less evident at 37° C., and the patient's whole blood clotting time was usually within normal limits at this temperature. Therefore, the influence of normal blood cells on the recalcified clotting time of the patient's plasma was tested (Table VIII). In these experiments, washed blood cells accelerated the clotting of both the patient's and normal plasma.

The patient's plasma contained a small amount of cryoglobulin. However, plasma from which the cold-precipitated cryoglobulin had been removed inhibited the clotting of normal recalcified plasma. In some of the patients reported by others the circulating anticoagulant behaved like an antibody. No procedures were performed to determine whether this was the case in the patient under study.

## DISCUSSION

The presence of a circulating anticoagulant is usually associated with remarkable prolongation of the patient's clotting time. In the present report a patient is described whose whole blood clotting time was prolonged at 25° C. but usually was normal at 37° C. The failure to demonstrate a prolonged clot-

TABLE VIII. THE EFFECT OF THE ADDITION OF NORMAL, WASHED 50 PER CENT SUSPENSION OF BLOOD CELLS ON THE CLOTTING TIME OF OXALATED PLASMA

Clotting time (min.) of mixtures of 0.1 ml. oxalated plasma, 0.1 ml. washed blood cells or buffer, and 0.1 ml. of 0.025M $CaCl_2$

| SOURCE OF PLASMA | TEMP. | CELL SUSPENSION | BUFFER |
|---|---|---|---|
| Patient | 25° C. | 6½ | 29 |
| Control | 25° C. | 4 | 8 |
| Patient | 37° C. | 3¾ | 11½ |
| Control | 37° C. | 2 | 3 |

ting time at 37° C. apparently was related to the presence of the formed elements of the blood, since the recalcified clotting time of oxalated plasma was prolonged at this temperature. Furthermore, it was possible to demonstrate that the addition of blood cells to the patient's plasma accelerated the recalcified clotting time, although, under the conditions used, the clotting time was not reduced to that of a control plasma similarly treated. These observations may provide an explanation for the fact that the patient did not demonstrate the usual hemorrhagic phenomena associated with delayed clotting and was able to undergo major abdominal surgery without abnormal bleeding.

The anticoagulant under study did not inhibit the action of thrombin nor did it appear to inactivate either fibrinogen or prothrombin. However, the conversion of prothrombin to thrombin in the presence of dilute thromboplastin was impaired. An anticoagulant with similar properties was described by Conley, Rathbun, Morse, and Robinson.[8] The manner in which this conversion was impaired is not clear. From the present data it is not possible to state whether the patient's anticoagulant exerted its effect by inhibiting thromboplastin or by interfering with such accelerating substances as Ac-globulin. The latter possibility seems less likely, since the patient's prothrombin time was normal at times when the circulating anticoagulant could be demonstrated. Proof that inhibition did not affect Ac-globulin was not possible because a supply of this substance was not available. Furthermore, it was not possible to determine whether, in addition, the anticoagulant inhibited the activation of plasma thromboplastinogen since impairment of the conversion of thromboplastinogen to active thromboplastin and inhibition of active thromboplastin both would be manifested by impaired conversion of prothrombin to thrombin. It is noteworthy, however, that in those instances recorded in which the circulating anticoagulant was thought to exert its effect against thromboplastinogen, the addition of decreasing amounts of thromboplastin disclosed no abnormality until the dilutions were much greater than in this study, and the clotting time was approaching that of recalcified plasma. It is evident that the anticoagulant in this study was different, in that the patient's prothrombin time was prolonged with undiluted thromboplastin at 25° C., and with a 1:10 dilution of thromboplastin at 37° C. This was true even in the presence of normal amounts of prothrombin, as was described.

The ability of the formed elements of the blood to counteract the action of the anticoagulant at 37° C. might be interpreted as the result of the release of potent thromboplastic material from the cells at this temperature. Which of

the formed elements was responsible cannot be stated, but leucocytes are known to have thromboplastic activity and platelets contribute in some manner to the activation of plasma thromboplastinogen.

The patient had evidence of increased capillary fragility and a prolonged bleeding time. These findings have been described in other patients with cryoglobinemia, and were probably unrelated to the circulating anticoagulant. Similarly, the hematuria was probably a manifestation of the primary disease, subacute bacterial endocarditis.

The frequency with which circulating anticoagulants have been reported suggests that tests for these substances should be performed in all patients with prolonged clotting times. Since anticoagulants have been described repeatedly in hemophiliacs, these patients should not be excepted from such studies. The demonstration of an anticoagulant active in whole blood at 25° C., but not at 37° C., suggests that the clotting time of whole blood should be determined routinely at room temperature.

## SUMMARY

In a patient with subacute bacterial endocarditis and cryoglobulinemia, the whole blood clotting time was prolonged at 25° C. but was usually normal at 37° C.

The prolonged clotting time was attributed to the presence of a circulating anticoagulant which was active in whole blood at 25° C. but not at 37° C. However, the anticoagulant was demonstrable at both temperatures in the absence of the formed elements of the blood.

The anticoagulant appeared to inhibit the conversion of prothrombin, an effect opposed by thromboplastin and by the formed elements of the blood.

We are indebted to Mrs. Rosemary Ashe for technical assistance.

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# CUTANEOUS ABSORPTION OF PHENOL

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THE analyses of casualties in the atomic bomb explosions in Japan show that 85 per cent of the casualties were a result of blasts and burns. Methods of treatment for such injuries have become of paramount importance. Many chemicals now deemed safe for use in the treatment of minor skin injuries should be re-examined for potential toxicity before extensive use is made in the treatment of such casualties.

Preparations containing phenol have been used topically as antipruritics and as antiseptic dressings for the treatment of burns, wounds, and indolent ulcers. On occasion such uses have been made with scant regard to the nature or extensiveness of the skin lesions involved. Deichmann,[1] in a recent review of the literature, cites a number of fatal intoxications following the topical use of phenol. There is ample evidence that phenol may be rapidly absorbed by the intact or injured skin in sufficient quantities to produce a fatal outcome.

Thus, our study was untaken to determine the comparative cutaneous absorption of phenol from bandages applied both to the intact and to certain types of damaged skin. The damaged skin was designated as abraded, burned with live steam (scald), burned with dry heat (heated iron), burned by ultraviolet light irradiation, and finally as exuding skin surface. The latter represented, as a rule, plasma exuding, denuded surface (skin burned by scald or dry heat in which the necrotic burned areas had been allowed to desquamate), largely devoid of any epithelial elements.

## METHOD

Albino rabbits weighing two and one-half to three kilograms were used in all experiments. Procedures involving traumatization of the skin were performed on animals anesthetized with sodium pentobarbital (25 milligrams per killogram intravenously). Exposures were made to an area, clipped free of hair, approximately 8 centimeters wide around the trunk of the animal. Abrasions were made by longitudinal incisions through the stratum corneum, two centimeters apart, on the area of exposure. Dry heat burns were produced by searing the area of exposure with a hot iron. Ultraviolet burns were produced by exposing the backs of the animals at a distance of 12 inches from an ultraviolet lamp for twenty minutes. The ultraviolet irradiations were made twenty-four hours prior to exposure to the phenol preparation. The source of ultraviolet irradiation used was a General Electric Uviarc portable A.C. Ultraviolet Outfit with an arc length of 6 inches, and giving 54 watts per arc inch. On one group of animals gauze bandages, on which a preparation containing 2.3 per cent phenol in corn oil was freely applied (without measurement), were placed around the area of exposure and secured by special wire screens.[2] This procedure insures against contamination of cage and food, as well as the possibility that the animal might ingest phenol. An unmeasured quantity was used to simulate the usual method of application as in the cases

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cited by Deichmann. For a more accurate comparison, a measured amount, 20 c.c., of the phenol preparation was applied to the gauze bandages and administered to another group of rabbits in a similar manner.

Bandages were maintained in contact with the skin for a continuous forty-eight hour period, then removed and the animal thoroughly scrubbed with soap and water to remove any residual phenol. Control exposures to intact and each type of damaged skin were made, substituting physiological saline for the phenol preparation.

Animals were kept in stainless steel metabolism cages and catheterized at twenty-four hour intervals. The catheterized sample of urine was added to the urine collected from the cage, thus enabling analyses of the total twenty-four hour urine samples. These urine samples were analyzed for phenol excretion during the period of exposure and daily thereafter until urinary phenol had returned to pre-exposure levels. The bulk of phenol excretion occurred during the forty-eight hours that the bandage was maintained on the animal. These values were therefore chosen for comparison.

Analyses for total phenol were made on 50 c.c. aliquots using Schmidt's[3] steam distillation method. Diazotized 5-nitro-2-amino-anisole recommended by Gomori[4] was used as the dye in place of the dye used by Schmidt in view of its greater stability. Phenol could no longer be detected in the distillates after collection of the sixth 100 c.c. fraction of any given sample. By this method 82 per cent of added phenol is recoverable. Furthermore, 64 per cent of the total recoverable phenol is consistently found in the first 100 c.c. fraction. For this reason phenol determinations were made on the first 100 c.c. of distillate and the total phenol value calculated as follows:

$$\frac{\text{Mg. of phenol determined in first 100 c.c. fraction of steam distillate}}{0.64 \times 0.82} = \text{Total amount of phenol}$$

The samples were compared in a Beckman spectrophotometer using phenol in water as a standard. This chemical assay is based upon the determination of hydroxyl groups, and thus includes phenols, cresols, etc., which are excreted after the administration of phenol.

Phenol is partially excreted in this manner and partially oxidized by the body. The portion excreted was used as an index of the amount absorbed. The possibility exists, of course, that in the traumatized animal the ratio of oxidized to excreted phenol is altered so that the amount excreted may not be a true index of the amount absorbed. Williams[5] has suggested that a shift in water balance affects phenol metabolism. In view of this, a decrease in the amount of phenol excreted by the animal with the burned skin might be expected.

## RESULTS

Table I gives the average values of urinary phenol in milligrams per forty-eight hour period excreted by exposed animals with intact and different types of

TABLE I. URINARY PHENOL EXCRETION PER 48 HOURS DURING A SINGLE CUTANEOUS APPLICATION OF 2.3 PER CENT PHENOL IN CORN OIL BY COMPRESS

| | COMPRESS WITH 20 C.C. OF MIXTURE | | | COMPRESS WITH MIXTURE FREELY APPLIED | | | COMPRESS WITH ISOTONIC SALINE SOLUTION | | |
|---|---|---|---|---|---|---|---|---|---|
| CONDITION OF SKIN | NO. OF RABBITS | MG. | STANDARD ERROR OF MEAN | NO. OF RABBITS | MG. | STANDARD ERROR OF MEAN | NO. OF RABBITS | MG. | STANDARD ERROR OF MEAN |
| Intact | 5 | 101 | ± 7 | 19* | 71 | ± 6 | 9* | 2.9 | ± 0.3 |
| Abraded | 6 | 91 | ± 18 | 18 | 80 | ± 9 | 10 | 3.1 | ± 0.3 |
| Ultraviolet irradiation | 9 | 109 | ± 7 | — | — | — | 11 | 3.8 | ± 0.4 |
| Dry burn | 10 | 141 | ± 17 | 11 | 105 | ± 11 | 6 | 1.9 | ± 0.3 |
| Steam scald | 11 | 133 | ± 16 | 10 | 119 | ± 17 | 6 | 2.1 | ± 0.5 |
| Denuded exuding surface following burn | 4 | 164 | ± 9 | 8 | 127 | ± 25 | 3 | 1.1 | ± 0.5 |

damaged skin. A comparison of the average excretion values shows no significant difference in absorption of phenol by the intact, abraded, and irradiated skin. The values for skin damaged by utraviolet light irradiation do not represent a true comparison, as the animals were irradiated on their backs only and not around the entire trunk. The highest value of phenol excretion was obtained from exposures to skin denuded following severe dry and steam burns. These denuded skin surfaces were actively exuding serous fluid, and in some cases there was also infection with pus present. The urinary phenol excretion values for the subjects so treated were slightly less than two times the values obtained in subjects with intact skin. The standard error is in some cases large. This is believed to be due to the difficulty in exactly duplicating the extent of skin trauma in animals.

Fig. 1 depicts twenty-four hour urinary phenol excretion during and following exposure. Maximal values for phenol excretion were obtained during the first twenty-four hours of exposure, indicating rapid absorption and elimination except in the case of the ultraviolet-burned skin. Although a characteristic pattern for excretion was obtained, the subjects with skin damaged by ultraviolet irradiation exhibited a delayed effect, in that high excretion values were obtained for the second day of exposure also. During the first twenty-four hours of excretion, the values were comparable to those obtained for the intact and abraded skin. However, higher values of excretion were obtained during the second day of exposure than was observed in any other type of damaged skin (Fig. 1). Regardless of the nature of skin damage, excretion values were down to control levels by the end of the third or fourth day of exposure. Normal phenol excretion values for the control subjects varied from 0.2 to 5.4 mg. per twenty-four hour period.

The phenol excretion values were highest for the first twenty-four hours of exposure as stated above. The significant drop in excretion values during the second twenty-four hour period, while the animal was still exposed to the phenol compresses could not be immediately explained. It may have been due to partial depletion of the phenol in the compress, or to a change in the character of the skin as an absorbing medium after twenty-four hours of exposure. To elucidate this observation, five rabbits with intact skin were prepared as before using 20 c.c. of the phenol mixture. At the end of the first twenty-four hours of exposure, twenty additional cubic centimeter of 2.3 per cent phenol in corn oil were added to bandages secured around the subjects. Average urinary phenol excretion values were found to be for the first day, 71; second day, 110; third day, 45; and fourth day, 4. The values for the second day of exposure were sufficiently high to indicate that the previous drop (Fig. 1) on the second day was due largely to depletion of phenol from the bandage.

*Camphor-phenol.*—A mixture of 5 per cent phenol and 10 per cent camphor in corn oil was prepared. This mixture was used in exposures in the same manner as the 2.3 per cent phenol in corn oil preparation. A series of exposures was made with both mixtures employing in all cases a total of 20 c.c. to each bandage for rabbits with intact skin. As shown in Table II the urinary phenol excretion values for the subjects exposed to camphor-phenol mixture were ap-

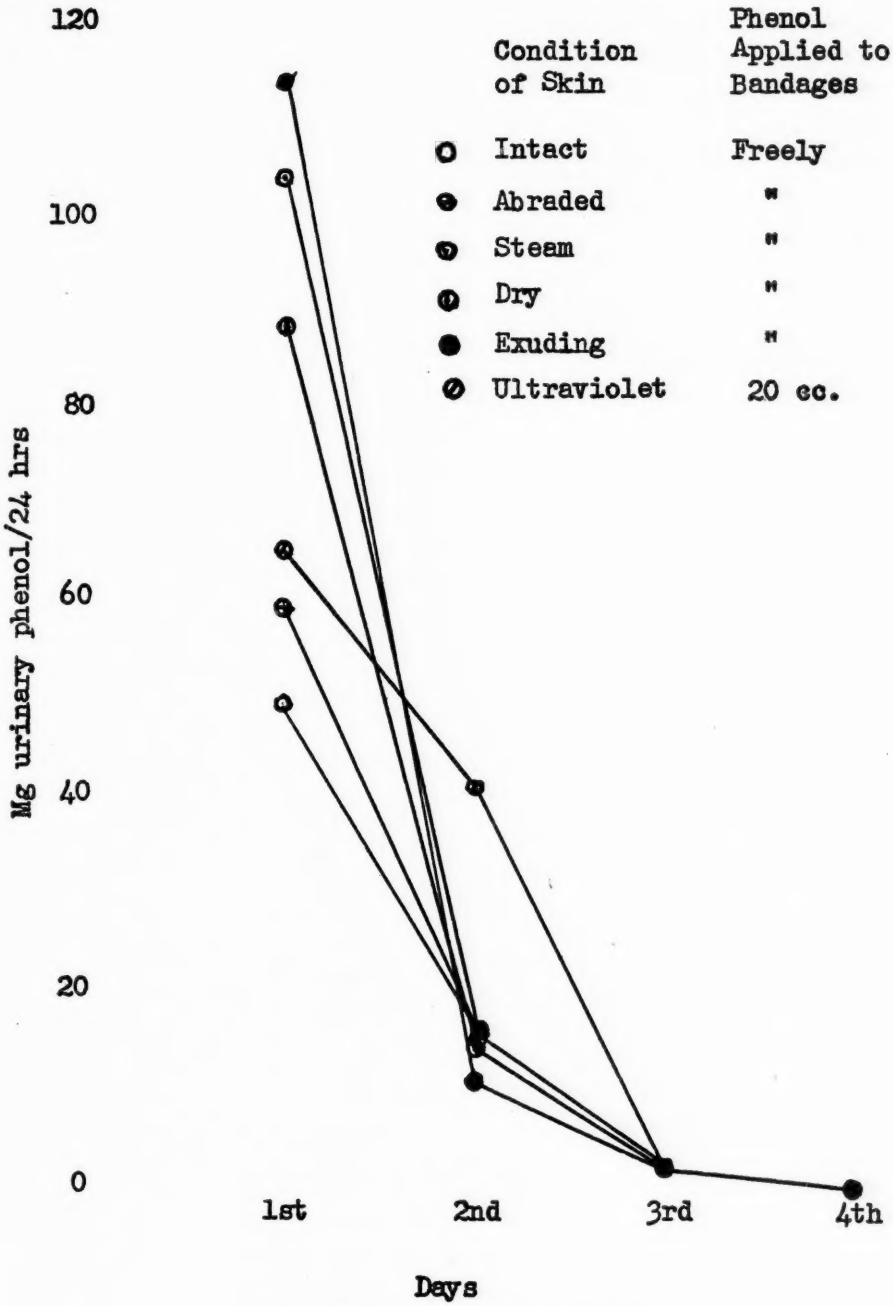


Fig. 1.—Daily urinary phenol excretion.

proximately twofold the value obtained for 2.3 per cent phenol in corn oil. This is in agreement with findings of Bass and Werch[6] that camphor does not affect the absorption of phenol from vegetable oil.

TABLE II. EFFECT OF CAMPHOR ON ABSORPTION OF PHENOL FROM CORN OIL

| | 2.3% PHENOL IN CORN OIL | | 5% PHENOL + 10% CAMPHOR IN CORN OIL | |
|---|---|---|---|---|
| | MG. OF URINARY PHENOL | NO. OF RABBITS | MG. OF URINARY PHENOL | NO. OF RABBITS |
| 1st day | 77 | 11 | 130 | 12 |
| 2nd day | 23 | 6 | 60 | 11 |

## CONCLUSIONS

1. Phenol is rapidly absorbed and excreted following topical application.

2. A single application of a 2.3 per cent phenol in corn oil mixture does not appear to affect the skin's ability to absorb phenol.

3. Animals whose skin was irradiated with ultraviolet rays to a point of erythema and slight edema formation exhibited a retarded excretion of phenol.

4. In the phenol-camphor and phenol-corn oil mixtures studied, urinary phenol values were in direct ratio to the phenol content of the respective mixtures.

5. After treatment, animals with severely burned skin exhibited much higher values of urinary phenol than animals with intact skin, indicating a potential danger in the indiscriminate use of phenol compresses in severe burns.

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# LABORATORY METHODS

## A MODEL DEMONSTRATING NORMAL AND ABNORMAL CARDIOVASCULAR DYNAMICS*

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A MODEL of the circulation which graphically illustrates cardiovascular hydrodynamics has been designed and used with success in teaching hemodynamics to undergraduate and graduate students of medicine, as well as in the analysis of problems in normal and abnormal cardiovascular hydrodynamics (Fig. 1). The model demonstrates certain aspects of Starling's law of the heart, and the effects of changes in the peripheral resistances. It permits first-hand observation of various factors affecting the circulation. It is constructed so that the progressive effects of various lesions leading to congestive circulatory failure, auricular and ventricular dilatation, pulmonary edema, and other consequences can be observed directly.

It is possible to analyze the effect on hydrodynamics of a single factor, while maintaining all other factors constant. For example, the dynamics of valvular insufficiency has been analyzed by assaying the effects of variations in stroke output, heart rate, auricular pressure, arterial pressure, arterial elasticity, and blood viscosity in order to determine the contribution of these factors to the hydrodynamics of the lesion.[1]

The model is constructed of glass and rubber. The driving force is supplied by compressed air through a specially designed valve.

### METHODS AND MATERIALS

*Auricle.*—The right and left auricles are similar in construction. Each auricle is composed of a truncated condom rubber of double thickness held in position by the cups of two thistle tubes of 38 mm. diameter. Rubber bands are used to tie the rubber portions in place over a cushion of folded penrose tubing. The venous return enters each auricle through a side arm in the inferior cup. A tube rising vertically for 36 cm. from the superior cup serves as a manometer recording auricular pressure. A second thistle tube cup is welded at the top of this manometer to serve as an antecubital vein for infusions into the system. The right auricular outflow passes out of the inferior cup via a side arm 7 mm. long, through a rubber or tygon tubing connection, to the equivalent of the tricuspid or mitral valves. The inferior cup has a second side arm which may be used in shunt experiments.

*Manometers.*—The manometer tubes representing auricular, ventricular, or arterial pressure pulses also may serve as reservoirs for fluid, depending on their cross-section area. This effect can be adjusted by the use of tubing of various diameters. For example, we have found it useful to use tubing of 5 mm. diameter for the intraventricular manometers and

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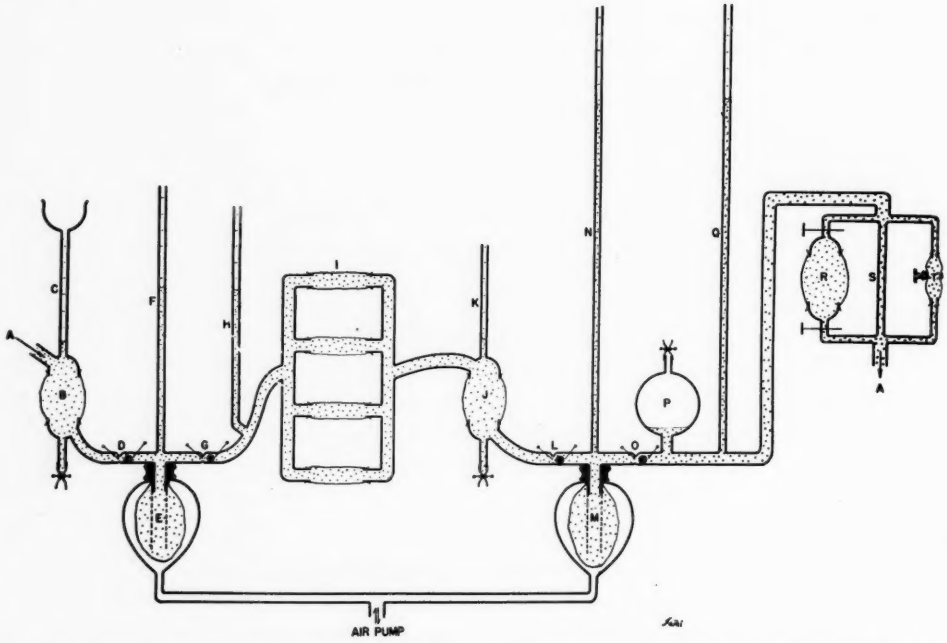


Fig. 1.—The venous return (*A*) enters the superior or inferior cup of the right auricle (*B*). Attached at its upper portion is a glass tubing which serves as an auricular manometer (*C*). At its top, the manometer is funnel shaped to permit additions of fluid to the circulation. The upper tube and all other manometers attached to the circulation are calibrated in units equivalent to 10 mm. Hg. Clamps similar to those on a Van Slyke apparatus hold the upper and lower tubes of the auricle, permitting upward or downward adjustment of the auricle and its tubes so as to vary the auricular pressure.

Fluid passes from the side arm of the inferior auricular cup via rubber or plastic tubing past the equivalent of the tricuspid valve (*D*) to the cross tube attached to the ventricular chamber. The valve (*D*) is described in detail in Fig. 2. The cross tube permits inflow into the condom serving as a ventricular chamber. The ventricle fills during diastole by virtue of auricular pressure. Filling is restricted by the elasticity of the ventricular wall, or by the ventricular "tone" arrangement described in the text. At the onset of systole, compressed air enters the pericardial glass chamber surrounding the ventricle causing it to contract and eject its contents. Since the ventricular wall may adventitiously obstruct the outflow tube, this path of egress is protected by the use of a piece of perforated flexible latex rubber tubing (*E*), having numerous small holes in it to insure free ejection.

The cross tube is held in the pericardial glass chamber by means of a rubber stopper fitting of the type used on serum bottles. This stopper closses the distance between the cross tube and the lip of the pericardial chamber. The arm of the cross tube passes through a perforation in the stopper and the lip of the rubber cap is pulled over the glass lip and secured in place by rubber band.

The upper limb of the cross tube may be used for connection of a manometer (*F*) to illustrate intraventricular pressure pulses, or for the introduction of an intraventricular shunt. In order to avoid the introduction of air into the circulation as a result of the fallaway of the intraventricular pressure to zero during diastole, a small glass tube sealed to the upper arm of the cross tube projects into the ventricular cavity.

The fourth limb of the cross tube permits flow to the pulmonary valve (*G*). Beyond the pulmonary valve, a water manometer visualizes the pulmonary arterial pressure pulses. The flow then passes into the lungs (*I*). The nature of this low resistance shunt is described in the text. Penrose or cellophane tubing is used as a connection between the glass parts of the lung apparatus.

Beyond the lungs, the fluid passes to the left auricle (*J*), the auricular manometer (*K*), the left ventricle (*M*), the left intraventricular manometer (*N*), and the aortic valve (*O*). These are similar in principle and construction to comparable parts of the right side of the circulation. Interposed between the aortic valve and the arterial pressure manometer is a section of penrose tubing (*P*) representing the elasticity of the arterial tree. This elasticity may be reduced in degree by restricting the expansion of the penrose tubing. The arterial pressure pulses are observed in a manometer (*Q*). The arterial outflow then passes to the systemic circulation where it may pass through channels *R*, *S*, or *T*. Channel *R* represents blood reservoirs such as those in the spleen and liver. Opening of the afferent stopcock permits inflow into the reservoir. Opening of the efferent stopcock permits addition of the stored fluid to the circulating volume. This reservoir is constructed in the same manner as the auricles, with a thin latex rubber tubing held in position on the superior and inferior glass cups by dyed rubber bands. Channel *S* represents a preferential pathway exhibiting little or no vasomotor tone. Increased systemic blood pressure results in a proportionate increase in flow through this channel. Channel *T* represents pathways with active vasomotor control as in the splanchnics, skin, and muscles. The resistance to flow through this channel is regulated by means of a screw clamp on a rubber tubing. The venous return from "*A*" enters the right auricular inflow tube at the left of the drawing (also labelled *A*). Further details are given in the text.

12 mm. for the arterial. By adjusting diameters of these tubes the pressure pulses and their volume effects may be increased or decreased. All manometer tubes in the model have a fine line of red or blue painted on the back. The thickness of this line should be about one-half the radius of the tube. When the water column rises, the lens action of the fluid causes the rising column to appear to be colored either red or blue. The front surface of the manometer tubes is graduated by white lines 13.6 centimeters apart, equivalent to 10 millimeters of mercury, to permit estimation of the pressure pulses during the cardiac cycle.

*Valves.*—Each valve is composed of a short glass tube constricted to about 6 mm. in its midportion (Fig. 2). One side of this constriction is ground (*G*) with pumice so that a 7.5 mm. stainless steel ball (*B*) can seat securely, obstructing backward flow. The outflow tract is composed of a cone of 2.5 mm. in length with a basal diameter of 9 mm. so that the obstruction at the outlet will not be greater than at the constriction. The cone is tilted so that the ball can seat without rolling up an incline.

A spring (*I*) mounted on a stainless steel stylet is introduced through the afferent rubber tubing. This may be advanced into position to prevent the steel ball from seating, thereby introducing graded degrees of valvular regurgitation. A similar stylet (*S*) protruding through the efferent rubber tubing has an attached spring which can be advanced to prevent free motion of the ball, producing graded degrees of valvular stenosis. Similar valve arrangements are utilized in appropriate positions as the tricuspid, pulmonary, mitral, and aortic valves. These valves easily withstand pressures of 300 mm. Hg for hours without appreciable leak. The back of the glass part is painted, if desired, with blue or red lacquer to simulate the presence of venous or arterial blood.

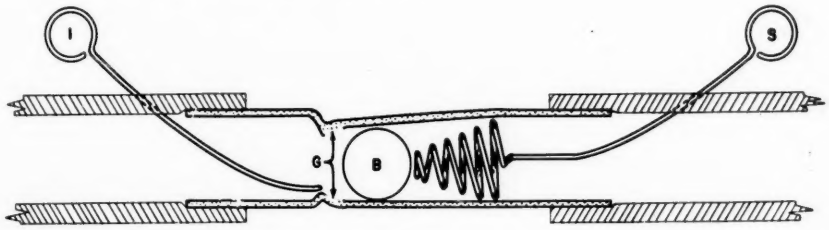


Fig. 2.—Detail of fluid valve. Heavy line: glass tube with a central constriction. The orifice, 6 mm. in diameter, is placed eccentrically so that the 7.5 mm. stainless steel ball (*B*) moves in a horizontal plane. Various degrees of insufficiency of the valve may be produced by advancing a stainless steel stylet (*I*) so that it prevents the steel ball (*B*) from seating properly in the ground glass seat (*G*). Since the outflow path is funnel shaped, varying degrees of valvular stenosis may be produced by advancing the stainless steel coil (*S*) so that the ball (*B*) progressively obstructs the outflow path. Combined insufficiency and stenosis of various grades may be produced by advancing both "*I*" and "*S*."
Construction of heart valve. Discussed in text under heading of *Valves*.

*Ventricle.*—The fluid passes from the valve via rubber tubing to a cross tube, and enters the ventricular chamber. The right and left ventricles are identical in construction. The ventricle is composed of condom rubber attached to the inferior arm of the cross tube.

Filling of the ventricle depends on the auricular pressure and on the duration of diastole. Factors resisting flow from the auricle include the resistance offered by the valve and tubing between the two chambers, and the elasticity of the ventricle itself. Resistance to filling of the ventricle can be modified by utilizing various thicknesses or duplications of the latex bag. The ventricle hangs in a pericardium composed of a 250 milliliter glass leveling bulb. This bulb is utilized for the transmission of air pressure to the outer wall of the ventricle, which causes ventricular contraction. The glass bulb acts similarly to the normal pericardium in that it provides an outer limit for ventricular dilatation. By introducing fluid into the bulb, it is possible to produce the effects of acute pericardial tamponade. Obviously, it does not yield like the normal pericardium to slowly accumulating effusions.

To insure complete emptying of the ventricle during systole, the lower arm of the glass cross tube has a piece of soft rubber of about 7 cm. length attached to it. This rubber tubing has small drilled holes of about 2 mm. in diameter to permit an adequate number of orifices for outflow if some should become obstructed. The intraventricular pressure manometer

extends into the rubber tubing to prevent the entry of air from the manometer during diastole. The outer walls of the ventricles may be stained with crystal violet for use as the right ventricle, or with Sudan III for use as the left ventricle.

*Motor.*—The mechanism is operated by air pressure. Eight pounds of pressure per square inch is sufficient to operate the entire apparatus. This can be supplied from compressed air lines or from a tank of compressed air. Compressed oxygen should not be used.

The heart rate is regulated by manual adjustment of a needle valve which permits compressed air to enter the windshield wiper motor (Trico No. FPS). The toggle arm of the motor opens and closes a specially constructed valve, which permits air pressure alternately to enter and then escape from the glass pericardium. The air valve, Crane three way steam cock No. 268, is bored with square holes to permit an abrupt transmission of air pressure so that maximal pressure for ventricular compression can be achieved within a short period. When the air valve is in the position of systole, air pressure is transmitted into the glass pericardium. In the position of diastole, the pericardial air is permitted to escape. The valve is constructed so that the relative duration of systole and diastole may be varied. The air valve is opened and closed by means of a pressure windshield wiper motor. The number of cycles per minute is adjusted by means of a needle valve permitting variation in the air pressure applied to the motor mechanism.

The toggle arm of the motor turns the inner core of an air valve. The outer shell of this air valve rises with the inner core until it is restricted at either end of its motion. One end of this movement is fixed by a permanent stop. At the other end, an adjustable stop permits limitation of the portion of the cycle during which the air pressure can be transmitted to the glass pericardial bulbs. In this way the duration of systole may be restricted within a constant cardiac cycle. The holes in the valve core are squared off to permit an almost instantaneous transmission of air pressure at the onset of systole. At the end of systole the exhaust orifice of the valve moves into position to permit rapid dropping of air pressure.

To increase the "tone" of the ventricle, the air from the glass pericardium about the ventricle is permitted to escape through an adjustable column of water. Thus an increased resistance to inflow into the ventricle apart from the simple hydrostatic pressure mechanisms is introduced into the system.

Intraventricular pressures are registered in glass manometers, 180 cm. long attached to the upper limb of the cross tube. At the onset of systole the air pressure inside the glass pericardial chamber begins to rise, exerting its force on the wall of the ventricle. The afferent valve closes when the intraventricular pressure is greater than the auricular pressure. As the pressure rises, the afferent valve is closed and then the efferent valve is opened, permitting ejection into the arteries.

During diastole, the intraventricular pressure falls to zero and air may enter the ventricle. This is avoided by having an extension of the intraventricular manometer extending into the rubber tubing attached to the lower limb of the glass crosspiece.

The energy available for ventricular contraction can be adjusted by means of a needle valve which regulates the air flow into the glass pericardia. A screw clamp on the air tube to the right glass pericardium may be used to reduce the energy available for this ventricle. The available energy for either ventricle may thus be adjusted at a level adequate for a given resistance to ventricular ejection. When the peripheral resistance is then increased and more energy is required for ventricular ejection because of the higher diastolic pressure, the ventricle will fail to empty completely. Over a period of time, dilatation may become marked with a transient increased pressure in the left auricle. A factor which makes this failure effect progressive depends on the fact that while the volume of the ventricle increases with the cube of the radius, the surface area available for the transmission of air pressure energy increases only by the square of the radius. Lowering of blood pressure by a reduction in the peripheral resistance is soon followed by recovery of the ventricle as shown by more adequate emptying. Opening of the valve to increase the delivery of air pressure to the pericardium simulates myocardial hypertrophy, or improved efficiency such as might occur with cardiotonic drugs.

A water manometer interposed between the pulmonary valve and the lung circuit permits visualization of the pressure pulses in the pulmonary artery, and serves as a reservoir for the pulmonary circuit. A similar manometer representing the systemic arterial pressure is interposed between the arterial elasticity air reservoir and the peripheral resistance.

*Lungs.*—The lungs are composed of two forked glass tubes connected by means of penrose or cellophane tubing to simulate the pulmonary capillaries. The inflow into the lungs passes along the fork handle into the tines, four or more horizontally placed glass tubes. The approximate height of the human lung is simulated by the distance between the upper and lower tines, about 35 centimeters. It can thus be shown that much of the right ventricular output passes through the more dependent pulmonary vessels. Flow through the upper portions of the pulmonary circuit occurs primarily during ventricular systole and whenever the left atrial pressure rises. Increased pulmonary resistance may be demonstrated by placing soft pinch clamps* on the capillaries. If it is desired to show pulmonary transudation, thin cellophane tubing is used for the pulmonary capillaries. The second glass fork of the lung collects the pulmonary flow and carries it to lower cup of the left auricle. The construction of the lungs permits the demonstration of the low resistances inherent in the normal pulmonary circuit.

*Arterial Elasticity.*—A volume elasticity effect is introduced into the arterial tree by having a portion of the arterial tree composed of several layers of penrose tubing. Elasticity of this system may be reduced by covering the penrose tubing with a layer of heavier tubing. An air reservoir may also be utilized, as described in Fig. 1.

*Peripheral Circulation.*—Outflow from the arterial tree depends on the head of arterial pressure and on the resistance to flow introduced by the model of the systemic circulation. In its simplest form the total peripheral resistance may be represented by a screw clamp on the rubber tubing leading from the arterial pressure manometer to the right atrium.

A more complete model of the peripheral circulation is composed of a central tube with two alternate channels. The central tube represents a preferential channel with minimal vasoconstrictor tone. In the model this is represented by a length of 7 mm. glass tubing with a lumen 1 mm. in diameter. Flow through this pathway depends primarily on the head of arterial pressure and on the resistances present in the alternative channels.

One side channel composed of a segment of rubber tubing and a screw clamp simulates those portions of the peripheral circulation with active vasoconstrictor tone, such as in the arterioles of the splanchnics, the skin, and the striated musculature. Closing of the screw clamp increases the resistance to flow through the channel.

The second alternative channel simulates the vascular reservoirs such as the liver and spleen. This is composed of upper and lower cups about 40 mm. in diameter connected by means of a latex central portion, similar to the construction of the auricles. Glass stopcocks are present above the superior cup and below the inferior cup. Opening of the superior stopcock permits flow into the expansible reservoir represented by the latex portion of the channel. This rubber part may be dyed red in its upper half and blue in its lower half. Fluid may be accumulated in the reservoir by closing the inferior stopcock. Opening the inferior stopcock empties the reservoir into the venous system.

Manipulation of the screw-clamp and stopcocks representing the adjustable peripheral resistances affects the arterial blood pressure. Thus an increase in the resistance to flow through the systemic circulation results in an increased head of pressure which becomes manifest in the arterial pressure column standpipe. As the pressure in the standpipe increases, an equilibrium is soon reached whereby the outflow becomes adjusted to the cardiac output.

*Artefacts.*—Attention must be called to certain artefacts. For example, an increase in the peripheral resistance results in a rise in the water level in the arterial manometer; the volume of fluid required to fill this portion of the manometer will thereby be withdrawn from the circulation as a whole, reducing the venous return. Reduction in the peripheral resistance results in a transiently increased venous return because of the reduction in the volume of fluid held in the arterial pressure standpipe. These artefacts may be reduced in degree by proper manipulation of the reservoir of the peripheral circulation.

*Commercially available curling pins may be used.

The introduction of an increased resistance to ventricular ejection results in an asynchrony of ventricular contraction which serves to emphasize the greater work done under these conditions.

The presence of water manometers throughout the system takes up some of the volume output, thus reducing the amplitude of the pressure pulses which would be obtained if the volume manometers were not present. This artefact may be diminished by using small bore tubing for the manometer tubes.

## MODIFICATION AND ATTACHMENTS

*Mounting.*—The apparatus is mounted on a masonite board, 4 by 6 feet and ½ inch thick. The clamps used are similar to those utilized in the usual Van Slyke apparatus.

*Color.*—The entire wood and masonite framework and the clamps were painted white. A stripe of blue or red scotch tape is pasted on the back of the glass tubing and on each of the manometers. This stripe is about one-half the radius of the tube. When the water column rises in the tube, the lens effect gives the illusion of a rising column of red or blue fluid. The latex and rubber tubing is colored by dipping into Sudan III for a red effect or into crystal violet for blue. Tygon is stained with lacquer in an alcohol base.

*Auricular Contraction.*—The auricular contribution to ventricular filling may be demonstrated by means of a second air valve operating at a fixed interval before ventricular contraction. The air blast is permitted to blow into a side arm of the auricular manometer, thus momentarily increasing the ventricular filling pressure. A more elaborate model consists of a glass chamber enclosing the auricle. In this case the blast of air is transmitted to the outer walls of the auricles. A separate air valve operating slightly ahead of the ventricular component is required for this demonstration. The time of auricular contraction may be varied with reference to the time of ventricular systole.

*Pericardial Constriction.*—The effect of constriction of the pericardium may be illustrated by enclosing the ventricle within a cellophane tube of proper size. This tube limits the ability of the ventricle to dilate during the filling phase, regardless of the auricular pressure or the duration of diastole. The effect of acute effusion may be produced by adding water or air between the cellophane and the ventricle, thus further limiting the ventricular diastolic capacity.

*Viscosity.*—Increases in the viscosity of the circulating fluid can be illustrated by the addition of small amounts of pectin to the fluid. A decreased viscosity may be produced by using warm water. The effect of viscosity changes is most easily illustrated in the presence of valvular lesions.[1]

*Cardiac Output and Blood Flow.*—Flows in any part of the model may be measured by the insertion of a flowmeter. The Ludwig stromuhr, rotameter, orifice, or bubble meter may be used. The rotameter has occasionally proved unsatisfactory since it may prevent reverse flow if the float impacts in the lower, constricted portion of the tube. Stroke output may be calculated from this value by dividing the minute output by the heart rate.

*Pulmonary Resistance.*—Resistance to flow through the pulmonary system may be increased by constricting the pulmonary capillaries with clips. This may also be done by increasing the air pressure within a glass or plastic cuff placed around the pulmonary capillaries, thereby collapsing them. In this way some of the acute effects seen in the Valsalva experiment or the chronic effects in emphysema and chronic cor pulmonale may be simulated.

*Pulmonary Edema.*—Utilization of thin cellophane tubing for the pulmonary capillaries permits the filtration of fluid through the walls of these vessels. This effect is noted especially in the dependent portions of the lungs, by virtue of the greater hydrostatic pressure at the bases.

*Constant Pressure Head.*—For some experiments it has been useful to differentiate the two types of resistance of the arterial system: (1) The quantum of energy produced by ventricular contraction before the aortic valve opens and ejection occurs, and (2) The resistance to outflow of blood from the arterial tree through the arterioles and capillaries (Poiseuille factor).

The first factor can be studied in a system which requires that the ventricular output flow over the top of an adjustable water column. In this system the Poiseuille factor remains unchanged and may be minimized by using wide tubing. The Poiseuille effect may be illustrated by using tubing of lesser diameter.

*Blood Volume.*—Variations in the blood volume can be produced by adding fluid to the funnel at the top of the right auricular manometer. The reservoir included in the systemic circuit also makes it possible to add or withdraw as much as 300 c.c. of fluid from the circulation.

*Failure.*—Some of the dynamic events associated with inability of the ventricle completely to eject the venous return may be illustrated by limiting the energy available for contraction. This is done by reducing the air pressure available for ventricular contraction. This effect may also be seen when marked hypertension is induced by an increase in total peripheral resistance, without providing an additional increment of air pressure energy for contraction.

*Shunts.*—A variety of shunts as listed below may be introduced into the circuit by utilizing side arms in each of the chambers or vessels. The degree of the leak may be adjusted with a screw clamp on the rubber tubing portion of the shunt.

*Heart Sounds.*—Sound produced by the apparatus may be heard by application of a stethoscope diaphragm to its various parts. Systolic or diastolic murmurs of various intensities are produced by introducing stenosis or regurgitation. Triple heart sounds occur in severe pulmonary or systemic hypertension.

The following hydrodynamic phenomena can be illustrated with the model:

A. Heart sounds and murmurs
   - normal heart sounds
   - systolic murmurs
   - diastolic murmurs
   - triple heart sounds

B. Pressure pulses
   - right auricular
   - right intraventricular
   - pulmonary arterial
   - pulmonary "capillary"
   - left auricular
   - left intraventricular
   - aortic

C. Aortic elasticity

D. Stroke and minute output

Hydrodynamic changes occurring on variation of the following can be illustrated:

E. Physiological variants
   - heart rate
   - blood pressure
     - pulmonary arterial
     - pulmonary venous
     - systemic arterial
     - systemic venous
   - blood volume
   - blood viscosity
   - cardiac "tone"
   - auricular contribution to ventricular filling

F. Pathological variants
   - hypodynamic heart
   - ventricular failure
   - valvular insufficiency
   - valvular stenosis

valvular insufficiency with stenosis
pulmonary congestion
constricted pericardium
shock
arteriosclerosis
coarctation of aorta
interauricular septal defect
interventricular septal defect
patent ductus arteriosus
left ventricular—right auricular shunt
right ventricular—left auricular shunt
aortic-auricular shunts
pulmonary artery-auricular shunts

SUMMARY

A model of the circulation which illustrates normal and abnormal cardiovascular hydrodynamics has been designed and used with success in the teaching of hemodynamics. Pressure pulses in any part of the circulation in a large variety of conditions may be demonstrated.

I wish to acknowledge the valuable and enthusiastic technical assistance in the development of this model of Hiroshi Saiki, B.S., Research Assistant; Francis Williams, B.S., Senior Research Technician; Emil Tiger, Chief Machinist, and Eugene Boyd, Glass Blower.

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# A SIMPLE ONE-TABLET TEST FOR URINARY CHLORIDE IN LOW SODIUM DIETS

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THE purpose of this paper is to present a modification of the urinary chloride test developed by Fantus[1] in which all ingredients are incorporated in one tablet, and to discuss briefly its clinical applications.

Van Slyke and Evans[2] have emphasized the importance of urinary chloride determinations in following salt depletion in human beings. Studying normal medical students made dehydrated and salt deficient by gastric lavage, they found that urinary chloride fell to zero on the second day of continuous suction. By this time, plasma chloride had fallen to only 571 mg. per cent (as NaCl), a decrease of only 6 per cent from control levels. At the height of the depletion period, on the fourth day, plasma chloride concentration had fallen only 24 per cent (463 mg. per cent), while the total NaCl loss was 32 Gm., twice as much as the normal total plasma salt content. During the correction of this salt deficient dehydration, urinary chloride reappeared as the plasma chloride level rose to 573 mg. per cent (as NaCl). As these authors state, "These figures show that the moderately severe salt depletion was quickly apparent from the decrease in urine chloride concentrations, while it was scarcely detectable from plasma chloride concentration determinations."

The value of urinary chloride tests in the diagnosis and treatment of salt and water depletion has been pointed out by Marriott,[3] in the treatment of pyloric obstruction dehydration by Sanchez-Vegas and Collins,[4] and in the bedside management of problems of fluid balance by Scribner, Power, and Rynarson,[5] and many others.[6-9]

During the course of low sodium diets, either the rigid rice diet of Kempner, his salt poor, fat poor modification, or other rigid salt deprivation programs, it is necessary to obtain frequent determinations of electrolytes to avoid excessive imbalance. Urinary chloride is a satisfactory guide to the electrolyte change in patients with normal kidney function. For example, on the Kempner rice diet, there is at first almost a linear decrease in urinary chloride. It reaches about 20 mg. per cent, on an average of one hundred days, varying from forty to sixty days to five or more months. Any marked change in the urinary chloride level indicates failure to adhere to the diet or a shift in the electrolyte pattern of the patient. The urine chloride concentration is almost constant at these low levels, so that a specimen taken at any time during the twenty-four hours will be representative of the daily chloride excretion as shown by Bryant and co-workers,[10] as well as Van Slyke and Evans.[2]

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The urinary chloride test is equally important during the period of additions to the low salt diet. By testing two to three times weekly, one can tell the rate at which the additions can be made.

The frequency with which the usual tests are made is a matter of debate, varying from twice weekly, especially at the onset of the program, to once in two weeks. The former is practical in institutional or semi-institutional management, but difficult on an out-patient basis. This requires a trip to the laboratory shortly before each visit, as well as imposing an additional financial burden.

Any attempt at supervising a low sodium or rice diet without adequate control is unsatisfactory and in some cases dangerous, hence it would obviously be desirable to have a simple urinary chloride test which could be performed in the office or even at home by reliable patients.[10]

## METHOD

The Fantus test is a semiquantitative determination based on the Mohr equation for halogen determination.[11] When silver nitrate is added to a solution containing chloride and potassium chromate, silver chloride begins to precipitate immediately. When the chloride is exhausted and the silver ion reaches a concentration of $7 \times 10^{-5}$ molar, it reacts with the potassium chromate to give silver chromate. This results in a color change from yellow to reddish brown. Heretofore, solutions of silver nitrate and potassium chromate have been used for urinary chloride determinations. These compounds were made in tablet form.* Later the silver nitrate and potassium chromate were incorporated in one tablet.† This simplifies and increases the ease of performance of the test and eliminates the danger of contamination of the solution.

There was no significant difference in the accuracy of the test done with solutions of $AgNO_3$ and $K_2CrO_4$, or by incorporating both reagents in one tablet when checked by the Van Slyke and Hiller[12] modification of the chloride determination devised by Sendroy.

A test tube, a dropper calibrated accurately to 20 drops per millimeter, chloride test tablets, and distilled water are all the equipment necessary.

To illustrate, using 5 drops of urine and 15 drops of distilled water: if one tablet is added (allow two to three minutes for disintegration) and the color changes from yellow to a reddish brown, the urinary sodium chloride is between 0 and 60 mg. per cent. If two tablets are necessary to bring about a color change, the value is between 60 and 120 mg. per cent. Tablets may be added until a total of five are used. The limits of solubility prevent further additions. It is to be pointed out that the color change may be from a slight pink to a reddish brown. The solution should be carefully examined after each tablet to see if the tablet has disintegrated and if a color change has occurred.

TABLE I. DIFFERENCE IN VALUES OF TABLETS BY CHANGE OF THE RATIO OF URINE TO DISTILLED WATER

| TABLETS* | 5 DROPS URINE 15 DROPS WATER | 3 DROPS URINE 17 DROPS WATER | 2 DROPS URINE 18 DROPS WATER |
|---|---|---|---|
| 1 | Less than 60 mg. % | Less than 100 mg. % | Less than 180 mg. % |
| 2 | Less than 60-120 mg. % | Less than 100-200 mg. % | Less than 150-300 mg. % |
| 3 | Less than 120-180 mg. % | Less than 200-300 mg. % | Less than 300-480 mg. % |
| 4 | Less than 180-240 mg. % | Less than 300-400 mg. % | Less than 480-600 mg. % |
| 5 | Less than 240-320 mg. % | Less than 400-800 mg. % | Less than 600-780 mg. % |

*Number of tablets necessary to bring about a color change from yellow to reddish brown.

The sodium chloride values of twenty urines were checked using the three dilutions in Table I on each and comparing it with the standard laboratory method. No decrease in

*Brunswick Laboratories, Chicago, Ill.
†Ames Company, Inc., Elkhart, Ind.

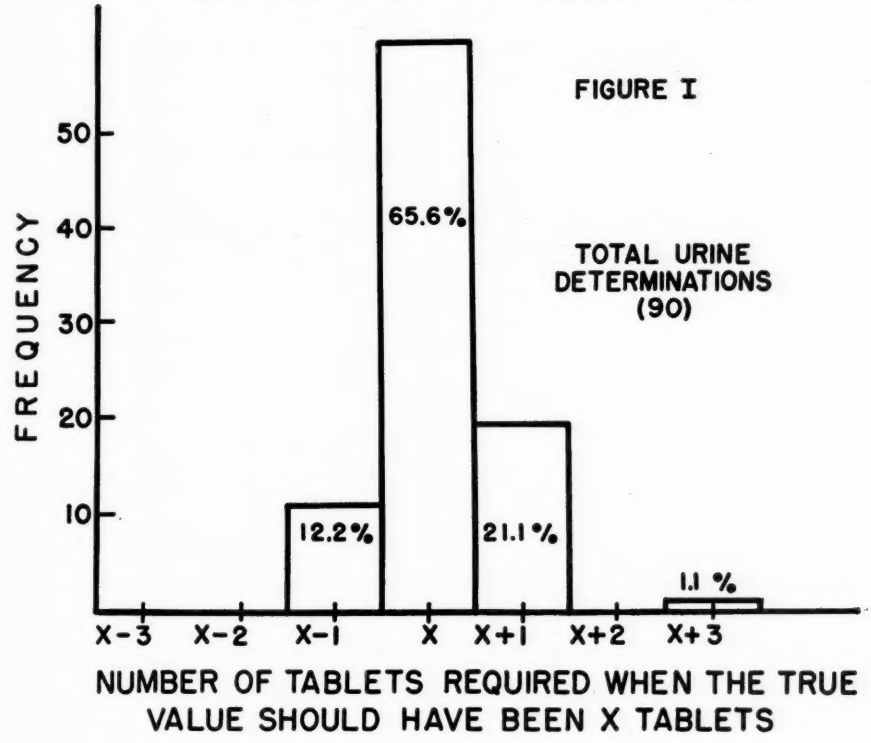


Fig. 1.

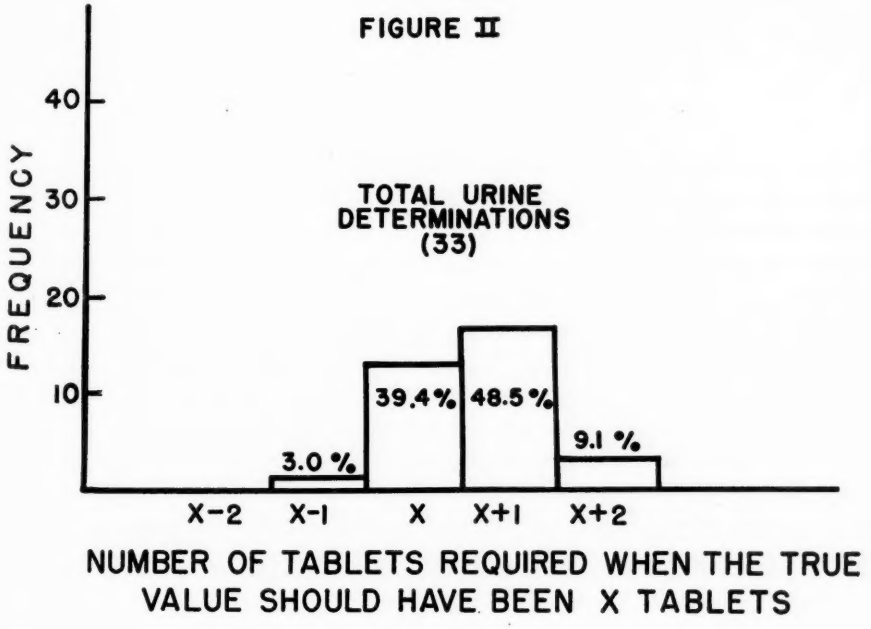


Fig. 2.

Figs. 1 and 2.—Results of comparing the accuracy of the chloride tablet test (5 drops urine and 15 drops water) with the standard laboratory procedure. The X column indicates that the laboratory determination is within the range determined by the tablet. The X + 1 deviation column indicates that the range is in error by one tablet more as compared to the standard laboratory procedure, the X – 1 deviation by one tablet less, etc.

accuracy was noted in the higher dilutions if care was used in the procedure. It is to be pointed out that the dropper should always be held in a vertical position, and distilled water must always be used.

The ratio of 5 drops of urine to 15 drops of water has been the most satisfactory in the management of hypertensive patients, since we were most interested in values of urinary sodium chloride below 200 mg. per cent.

The various inorganic compounds normally found in the urine were titrated against a 0.017 molar solution of $AgNO_3$, using potassium chromate as an indicator. Solutions of $Na_2SO_4$, $KH_2PO_4$, $Na_2HPO_4$, and $K_2HPO_4$ were used in concentrations equal to or greater than those usually found in the urine. Solutions containing the $PO_4$ ion were the only ones which react with $AgNO_3$.

However, these values were only a fraction of the total blank values obtained when urines were titrated against $AgNO_3$ and checked by the standard laboratory chloride method. It is known that pH and solubility effect this reaction; the exact effect of other complex factors is poorly understood.[11] The average total blank values were compensated by additional $AgNO_3$ in each tablet. The blanks are allowed for in the calculations in Table I and Figs. 1 and 2.

## RESULTS

One hundred twenty-three specimens were used to check this method with the standard laboratory method. They were obtained from routine hospital admissions, outpatients on the Kempner rice diet, and from patients receiving ACTH, who were on a low sodium diet with additions of KCl.

Fig. 1 includes all values up to 300 mg. per cent, and Fig. 2 all values over that. If, for example, the standard laboratory determination was 150 mg. per cent and the determination by the tablets was between 120 and 180 mg. per cent, this would be placed in the X column; if the determination by the tablets was 180 to 240 mg. per cent, it would be placed in the X +1 deviation from the true interval column; and if 60 to 120 mg. per cent by the tablets in the X –1 deviation column.

## COMMENTS

Most of our experience in the use of this test has been in the management of hypertensive patients on low salt diets. One of us has been satisfactorily managing hypertensive patients in his office for the past nine months. With a minimum amount of instruction most patients are able to satisfactorily test their specimens at home and report their results by telephone or at office visits. Also of importance, they can themselves follow their salt balance while on vacation, since most dietary errors occur when eating in restaurants, hotels, or as guests. Faults can easily be detected by the patient, allowing him to travel with a minimum of fear and maximum control of his program.

We are led to believe that this test is valid in patients (a) with normal kidney and cardiac function, (b) with renal or cardiac damage but who are compensated. This includes the patients who may have normal or slightly decreased kidney function tests, but who are excreting an adequate amount of chloride and urine without clinical evidence of edema.

It is our impression that this test is not valid when there is uncompensated cardiac or renal disease, or clinical evidence of edema. In any borderline cases more extensive determinations of the blood and urine electrolytes should be done to adequately determine the status of the patient.

Limited use in the handling of surgical patients has proved satisfactory. We have followed the program reported by Van Slyke and Evans.[2] The essential features of this program are as follows: accurate twenty-four-hour urine and chloride output. These determinations can be made at the bedside on morning rounds and replacement therapy planned on the basis of the findings. They conclude that water and salt balance will be properly maintained if daily urine volume exceeds 1,500 ml. and urine salt concentration approximately 3 Gm. per liter, providing kidney and adrenal function are normal. A more detailed program was recently reported by Scribner, Power, and Rynearson.[5] We feel that our test could be used with either method of control.

The following limitations should be remembered in using this test: (1) The value of this test is based on the assumption that chloride metabolism for practical purposes, is similar to sodium metabolism. In infrequent instances this may be misleading. (2) Urinary chloride serves as a satisfactory guide in the management of the aforementioned conditions, but does not indicate the status of electrolytes such as potassium, calcium, magnesium, etc. (3) Very infrequently we find that this urinary tablet test may be inaccurate (see Figs. 1 and 2 and Reference 11).

## CONCLUSIONS

If the limitations of the test are kept in mind, it can be an important adjunct in the management of fluid and electrolytic balance.

We consider that its greatest use will be as (1) an intermediate procedure between the more extensive electrolytic determinations in patients requiring very close control and (2) as a guide to management of outpatients on low sodium diets.

## SUMMARY

A simple one-tablet urinary chloride test is presented in which, if a ±1 tablet deviation is allowed, over 95 per cent of the determinations fall within the range of the standard laboratory determination. Fewer deviations occur in the range below 300 mg. per cent than above. The clinical applications are briefly discussed.

We would like to express our appreciation to Dr. Alma Hiller for her aid and many helpful suggestions; to Miss Helen Naska for her technical assistance; and to Mr. Gilbert H. Scribner for the grants which made this work financially possible.

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# AN EXPERIMENTAL STUDY OF RESPIRATION MAINTAINED BY HOMOLOGOUS LUNGS

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AS CARDIAC surgery has developed, increasing interest has been stimulated in extrapulmonic introduction of oxygen into the system, and in mechanical methods of respiration. Our attempts, as well as those of others, to increase oxygenation by intravenous administration or by transperitoneal absorption have met with failure. The amounts of oxygen which can be given safely are too small to be of recognized value. Gibbon,[1, 2] Björk,[3] Jongbloed,[4] Dennis,[5] Clark,[6] and others have worked on the problem of constructing artificial lungs which will accomplish complete gaseous exchange for considerable periods of time.

The idea of maintaining respiration with homologous lungs presented itself during a discussion of means of oxygenation from this chance remark: "Why not attach a lung to a dog's leg?" Well, why not? Consequently, a technique has been evolved whereby anesthetized dogs have been kept alive two to three hours entirely on the lungs of another dog. This report is made only to record the fact that homologous lungs are effective respiratory organs. The possibility of practical application is dimly suggested, but it must not be assumed that respiratory failure can be supported by pinning a lung on the sufferer's shoulder. The experimental procedure, still rather crude and not even done under aseptic conditions, does however suggest a number of studies in the basic subject of respiration.

## TECHNIQUE

Two dogs of about the same size, weighing approximately 20 pounds each, are chosen and matched for blood compatibility.

Dog A, the donor, is anesthetized with nembutal and is given 10,000 units of heparin* intravenously. The chest is widely opened by splitting the sternum longitudinally. The dog, of course, promptly dies as the chest is opened. The azygous vein, the superior and inferior vena cava, and the arch of the aorta are isolated, doubly ligated, and cut between the ligatures. The trachea is drawn down and cut leaving a long stump attached to the lungs. The heart and lungs are now carefully freed from their attachments in the chest and removed en masse. The main pulmonary artery is thoroughly isolated at the base of the heart and clamped. Another clamp is placed near the bifurcation. The pulmonary artery is then severed between the clamps, leaving as long a segment of the pulmonary artery as possible. The pulmonary veins are clamped, and the heart is cut away except for the left auricle. The end of a glass tube about 4 inches long and of proper size is tied into the pulmonary artery. A de Pezzer catheter with all but the flange removed is placed in the left auricle and fixed with a purse-string suture snugly tied around

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*The heparin was furnished by the Upjohn Pharmaceutical Company, Kalamazoo, Mich.

the catheter. It is important that the clamps on the pulmonary artery and the pulmonary veins be left closed while these tubes are being placed so that air may not enter the circulatory system.

The preparation, consisting of intact lungs, trachea, pulmonary artery, and pulmonary veins with properly placed tubes is now set aside and kept moist with saline solution.

The large blood vessels in the dead dog are opened, and the expressible blood is aspirated and placed in a basin for later use.

Dog B, the recipient dog, is now anesthetized with nembutal, 1 c.c. of a 1 per cent solution per 5 pounds of weight, and ⅛ grain morphine sulphate, and 1/100 grain atropine sulphate given intravenously. A tracheotomy is done, and a well-fitting rubber tube is inserted into the trachea and snugly tied.

The abdomen is opened through a low right rectus incision. The aorta and inferior vena cava are isolated proximal to their level of bifurcation. At this point 10,000 units of heparin are injected into the inferior vena cava. Glass tubes of proper size are tied into the aorta and inferior vena cava. By means of long rubber tubing, connections are established with the lung preparation, the aorta being connected with the pulmonary artery, and the pulmonary veins with the inferior vena cava (Fig. 1). Again it is important while completing the connections to eliminate all air from the circulatory system.

Sufficient blood, previously collected, is pumped into the dog through the rubber tubing to replace what has been lost and to compensate for filling the tubes and the lung preparation.

We have inserted a glass T tube in the rubber tube going from the aorta to the lungs, and have connected it with a mercury manometer for continuous blood pressure recording.

An intratracheal tube is inserted into the trachea of the lung preparation, and to it is attached an anesthetist's soda lime cannister, oxygen tube, and breathing bag.

A small balloon filled with nitrogen is tied over the end of the tracheotomy tube of Dog B, the recipient. This balloon allows the dog to make respiratory movements, but does not allow any exchange of gases.

(Reference to the dog henceforth refers to the living animal, Dog B, or the recipient. The word "breathing" in quotation marks refers to manual respiration in the lungs removed from Dog A, the donor.)

"Breathing" in the lung preparation is immediately begun and maintained by rather rapid intermittent pressure on the anesthetic bag. The elasticity of the lungs is sufficient to collapse the lungs when pressure on the bag is released and thereby to provide to-and-fro movement in the soda lime cannister for absorption of carbon dioxide.

Within a couple of minutes after "breathing" has begun, dark, cyanotic blood can be seen coming from the aorta and pink, oxygenated blood going into the inferior vena cava. If there is any blood loss it is made up by injecting 50 c.c. of blood from time to time. After the technique was perfected this procedure has been repeated consecutively on six pairs of dogs without failure. The protocols of two experiments are briefly reviewed.

## PROTOCOLS

EXPERIMENT 5.—March 6, 1951, at 10:50 A.M. the lungs of an anesthetized dog weighing 27 pounds were removed as outlined. Dog B, the recipient, was anesthetized and prepared. At 11:50 A.M. "breathing" was begun. In a very few minutes the dog's respiratory attempts became feeble, infrequent, and finally consisted of barely visible chest movements. At 12:50 P.M., one hour after "breathing" had been commenced, the dog ceased completely to make any respiratory motion. His chest remained completely inactive for the next hour and forty minutes. At 2:30 P.M., after the dog had been carried on the respiration of homologous lungs for two hours and forty minutes, "breathing" was stopped to see whether the dog would again make respiratory attempts. Perhaps we did not wait long enough to allow the carbon dioxide of the blood to build up sufficiently high to stimulate

respiration, or it may be that the respiratory center becomes less responsive after a long period of disuse. Although the animal became visibly cyanotic, he made no attempt at respiration.

"Breathing" was again instituted and continued for another half hour. For some reason when "breathing" was stopped this time the dog began to breathe spontaneously. The nitrogen-filled bag was removed from his trachea and he was allowed to live until 4:45 P.M., when it seemed useless to prolong the experiment further.

EXPERIMENT 6.—March 9, 1951, at 10 A.M. the lungs were removed from an anesthetized dog weighing 18 pounds and prepared as above. Dog B, the recipient, was anesthetized and all connections made with the homologous lungs. At 11:07 A.M. "breathing" was begun (Fig. 2, *A*). Within *one* minute the dog stopped making any attempts at respira-

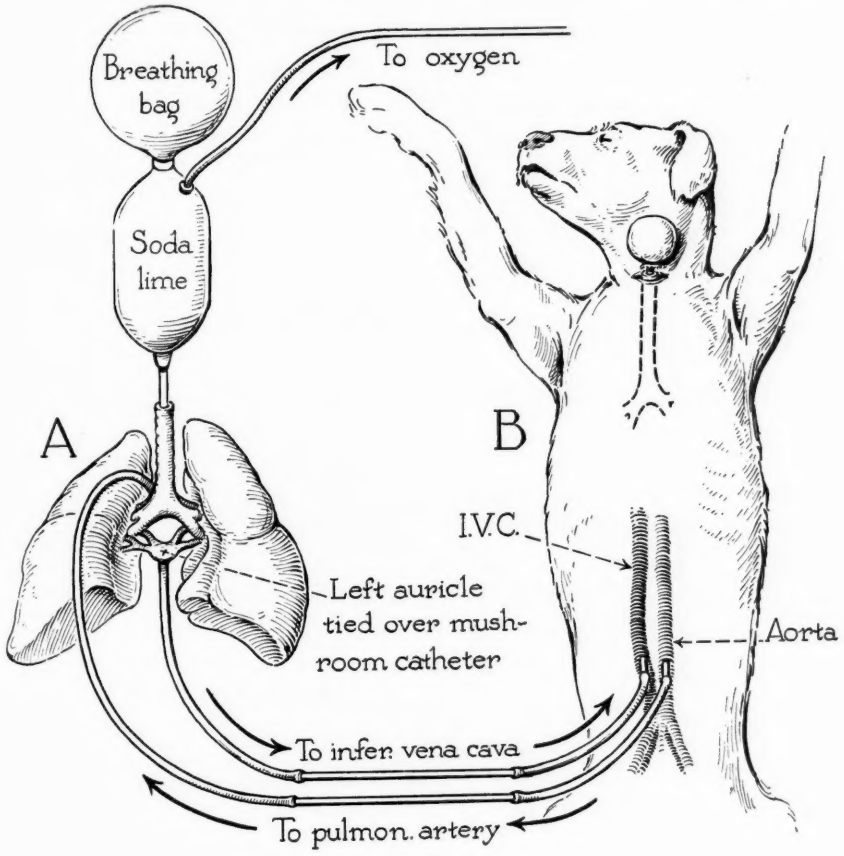


Fig. 1.

tion. His blood pressure and pulse remained normal. At 11:25 A.M. "breathing" was stopped (Fig. 2, *B*). After three minutes of complete apnea, respiratory attempts began and soon became very deep and labored. The blood pressure and pulse remained normal during this period of apnea. As soon as the animal showed signs of asphyxia "breathing" was again begun. Within twenty seconds the dog again ceased to make attempts at breathing. At 1:05 P.M., approximately two hours after respiration had been taken over by the homologous lungs, "breathing" was again stopped. This time the tubes leading to and from the lung preparation were clamped (Fig. 2, *C*). Immediately the dog began to try to breathe. After five minutes of no exchange of gases respiratory efforts ceased, but the pulse con-

tinued to beat, although slowly and irregularly. At the point it appeared that the dog was about to die, "breathing" was vigorously resumed. The blood promptly changed from black to pink, and in a few minutes the pulse and blood pressure had been restored to normal. The experiment was terminated.

## DISCUSSION

In the finest microscopic unit of the lung there is a single cell separating air from blood. Whether this epithelium has a specific respiratory function or whether it acts merely as a mechanical structure which allows the interchange of gases has long been discussed and decided in favor of the latter premise. These experiments would appear to support that conclusion.

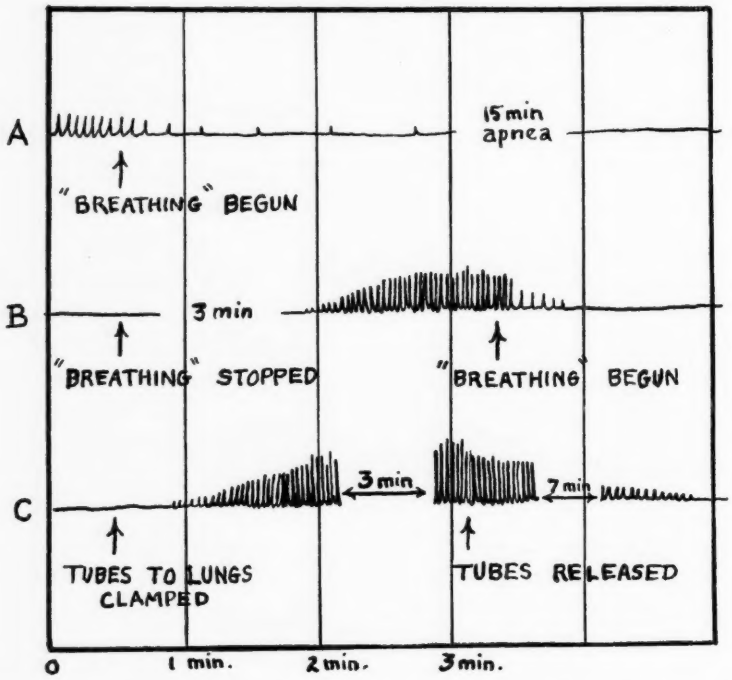


Fig. 2.—Spirogram, Experiment 6. *A*, Apnea occurred one minute after "breathing" with homologous lungs was begun and persisted for eighteen minutes. *B*, Three minutes after "breathing" was stopped the dog made respiratory attempts. When "breathing" was resumed the dog again promptly became apneic. *C*, When the blood flow to the homologous lungs was interrupted the dog began to make respiratory attempts. After five minutes when the animal seemed at the point of death, the tubes were released and vigorous "breathing" was resumed. The dog's condition quickly improved and after eight minutes he again ceased to make respiratory attempts.

In the early experiments "breathing" was carried on in the lung preparation continuously while the recipient dog was being prepared. In the last two experiments the lungs were simply laid aside for the hour necessary to prepare the second dog. No difference in the effectiveness of the lungs could be detected. In two experiments extensive pulmonary edema developed in the lung preparation after an hour and a half of "breathing." Much frothy material was aspirated from the trachea. In the lung preparation which was used for breathing for over three hours no pulmonary edema developed.

We were more intent upon determining the feasibility of keeping a dog alive on the lungs of another and upon the technique involved in accomplishing this than upon the chemical changes in the blood. However, a few determinations showed that the oxygen content of the blood coming from the lung preparation was that of arterial blood, varying from 20 to 25 per cent, and that of the blood going to the lung preparation was low, varying from 14 to 20 per cent. The carbon dioxide content of both bloods showed the same variations, but both were below normal. This subject and the Ph of the blood require further study.

A series of experiments is planned in which all of the blood from the superior and inferior vena cava will be pumped through homologous lungs and back into the dog's aorta, completely by-passing the heart. Since the dogs in these experiments were able to live while only the blood from the distal aorta (approximately one-fifth of the total blood flow) was being pumped through the lung preparation, it appears likely that they can be maintained for long periods of time when all the blood is being pumped through the homologous lungs.

## CONCLUSION

In six experiments it has been demonstrated that a dog not allowed to breathe with his own lungs can "breathe" for at least three hours by deflection of part of his blood through homologous lungs.

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# THE QUANTITATIVE MEASUREMENT OF EVANS BLUE (T-1824) AND ACID HEMATIN IN TISSUE HOMOGENATES

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IN STUDIES of iron and pigment metabolism it is often desirable to obtain either hemoglobin or one of its derivatives in a pure state. This is particularly true when radioisotope tracers are used in hematological studies, and it is necessary to separate hemoglobin from related compounds in homogenates of tissues involved in hematopoiesis. The purpose of this paper is to present a relatively simple method for the quantitative determinations of Evans Blue and acid hematin in tissue homogenates.

## METHOD

Tissue homogenates are prepared and diluted to known volume from organs obtained from normal adult white rats of the Wistar strain. The homogenizations are performed in distilled water in Waring blenders surrounded by an ice bath. One milliliter of tissue homogenate containing Evans Blue is placed into a calibrated and graduated centrifuge tube (capacity 15 milliliters). This is thoroughly mixed with 3.0 milliliters of alcoholic phosphotungstic acid reagent prepared in the manner described by Crooke and Morris.[1] Approximately 5.0 milliliters of ether are then added. If separation into a clear aqueous and ether phase does not occur promptly, this separation can be produced by adding a small volume (about 3.0 milliliters) of distilled water. The sample is centrifuged at 2,500 revolutions per minute for five minutes. The ether extracts all of the acid hematin, leaving a clear and distinct aqueous phase which contains the Evans Blue. The amorphous residue forms a small precipitate at the bottom of the centrifuge tube. The volumes of the aqueous and ether phases are recorded. A portion of the ether phase is rapidly transferred to a small cuvette, tightly stoppered to prevent evaporation, and read at 400 mμ in a spectrophotometer to determine its acid hematin content. Similarly a portion of the aqueous phase is measured at 610 mμ for its T-1824 content.

A knowledge of the original homogenate volume, the Evans Blue dye aliquot volume and the acid hematin ether aliquot volume provides the data for calculating the original homogenate content of T-1824 and acid hematin.

## RESULTS

The organs studied included lung, liver, kidney, spleen, and intestine.

Table I shows the results of five aliquot samples analyzed consecutively from each tissue homogenate when the quantity of added T-1824 was known. The dye recovery ranged from 86.5 per cent for the liver, to 103 per cent for the kidney. Except for the liver the yields were in excess of 90 per cent. The small standard deviations for both the T-1824 and acid hematin determinations indicate the consistency of the method.

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## HEMATIN IDENTIFICATION

The identity of the chromogen extracted into the ether phase was established by the following methods:

1. A known sample of acid hematin was treated with the alcoholic phosphotungstic acid reagent and then dissolved in ether. This sample was placed in a Cary recording spectrophotometer and a record of its characteristic absorption peaks between 400 and 700 mμ obtained. Chromogens extracted from

TABLE I

| ORGAN | ALIQUOT | MG. T-1824 IN HOMOGENATE | UNITS HEMATIN IN HOMOGENATE | % T-1824 RECOVERED | S. D. T-1824 | S. D. HEMATIN |
|---|---|---|---|---|---|---|
| Kidney | A | 1.33 | 18.68 | 103.0 | 0.013 | 0.328 |
| | B | 1.35 | 17.30 | | (0.95%) | (1.84%) |
| | C | 1.38 | 17.07 | | | |
| | D | 1.38 | 18.19 | | | |
| | E | 1.39 | 17.84 | | | |
| Spleen | A | 1.27 | 31.01 | 96.2 | 0.0056 | 0.845 |
| | B | 1.29 | 29.25 | | (0.43%) | (2.69%) |
| | C | 1.27 | 31.55 | | | |
| | D | 1.29 | 33.91 | | | |
| | E | 1.29 | 30.78 | | | |
| Lung | A | 1.26 | 18.89 | 93.2 | 0.013 | 0.286 |
| | B | 1.23 | 17.89 | | (1.01%) | (1.51%) |
| | C | 1.24 | 19.26 | | | |
| | D | 1.20 | 19.10 | | | |
| | E | 1.26 | 19.26 | | | |
| Liver | A | 1.14 | 35.30 | 86.5 | 0.0158 | 0.657 |
| | B | 1.13 | 34.76 | | (1.37%) | (1.88%) |
| | C | 1.12 | 36.93 | | | |
| | D | 1.14 | 33.54 | | | |
| | E | 1.20 | 34.03 | | | |
| Intestine | A | 0.912 | 17.91 | 92.6 | 0.061 | 0.50 |
| | B | 0.912 | 16.67 | | (0.68%) | (3.04%) |
| | C | 0.888 | 16.57 | | | |
| | D | 0.888 | 15.37 | | | |
| | E | 0.895 | 15.65 | | | |

One unit of hematin is arbitrarily expressed as that quantity of the ethyl ester of acid hematin which in 1.0 c.c. of ether reduces the light transmission by 90 per cent at 400 mμ.
S. D. = Standard deviation.
Figures in parentheses express S. D. as per cent of mean.

whole blood and from a liver tissue homogenate by the method described in this paper were also analyzed by the recording spectrophotometer. The tracings were identical.

2. Diluted portions of acid hematin, blood chromogen and liver tissue chromogen were treated with the alcoholic phosphotungstic reagent and then dissolved in ether. The samples were treated with a 5 per cent solution of sodium bicarbonate to extract the phosphotungstic acid and then evaporated to dryness. The dry residue was dissolved in butyl alcohol. Paper chromatographic analyses revealed homogeneity of each sample with almost identical Rf migration rates. The Rf values for "acid hematin," blood chromogen and liver tissue chromogen were 0.88, 0.90, and 0.89, respectively. The data strongly suggest that the chromogen is primarily the diethyl ester of acid hematin.[2]

## DISCUSSION

The alcoholic phosphotungstic acid reagent is unstable when exposed to the light. Therefore it should be kept in a bottle covered with black photographic paper and stored in a dark cool place. When cared for in this manner the reagent remains stable for at least two weeks.

Tissue homogenates should be analyzed for their Evans Blue content on the day of preparation. Overnight storage at 5° C. does not prevent the degradation of T-1824. Refrigeration at –25° C. for twenty-four hours prevents any significant loss of T-1824.

## SUMMARY

A simple technique for the simultaneous extraction and quantitative measurement of T-1824 and acid hematin in tissue homogenates has been described.

The authors wish to express their appreciation to Dr. David Howton for the Cary spectrophotometer records, and to Mr. Randolph Norton for the chromatographic analyses.

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# TURBIDIMETRIC METHOD FOR DETERMINING SENSITIVITY OF BACTERIA TO ANTIBIOTICS

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IN THE course of studies which were concerned with an evaluation and comparison of some of the practical methods available for determining the sensitivity of bacteria to antibiotics, it was of interest to investigate the feasibility of employing a turbidimetric or electrophotometric method for this purpose. Turbidimetric methods have been used to a considerable extent for the assay of antibiotics, chiefly during production operations and to a less extent in clinical investigations.[1] The use of such method for determining the sensitivity of bacterial strains has been very limited.[2] This paper deals with some of the limitations and problems involved in the application of a turbidimetric method employing a spectrophotometer for determining the sensitivity of bacteria to some of the antibiotics that are in common use.

## MATERIALS AND METHODS

*Organisms.*—The following organisms were used: *Salmonella typhosa,* phage type B1; *Salmonella paratyphi* B; *Shigella dysenteriae* I; *Bacillus cereus,* No. 5; *Streptococcus* 98; *Klebsiella pneumoniae,* T, and *Staphylococcus aureus* 3. The strains of Salmonella were obtained from Dr. P. R. Edwards of the Enteric Bacteriology Laboratory, and the Shigella strain from Dr. W. H. Ewing of the Shigella Unit, both of the Communicable Disease Center, United States Public Health Service, Chamblee, Georgia. *B. cereus* No. 5 was obtained from Dr. B. M. Duggar, of Lederle Laboratories, anl *Streptococcus* 98 from Dr. C. S. Keefer. The latter two strains and the Klebsiella, which was a type A strain isolated from sputum, have been in use as stock strains in this laboratory for assay of antibiotics. *Staphylococcus* 3 was a coagulase positive, hemolytic strain recently isolated from a purulent otitis media. None of these strains had previously been exposed to antibiotics. The cultures were maintained by repeated transfer in brain-heart infusion broth (Difco), pH ± 7.4 or on the surface of heart infusion agar (Difco) pH ± 7.4, 10 per cent defibrinated equine blood being added to the latter for the streptococcus and staphylococcus cultures.

*Antibiotics.*—The following agents were used: crystalline potassium penicillin G (Commercial Solvents Corp.), 1,595 units per milligram; streptomycin calcium chloride complex (Merck); crystalline aureomycin hydrochloride, furnished by Dr. S. M. Hardy of Lederle Laboratories; chloramphenicol (Chloromycetin), provided by Dr. E. A. Sharp of Parke, Davis & Co., and crystalline terramycin hydrochloride obtained from Dr. G. L. Hobby of Chas. Pfizer & Co.; Neomycin sulfate, supplied by Dr. E. L. Burbidge of The Upjohn Co. was also used with some of the organisms. Solutions of these antibiotics were freshly prepared each time before use, the concentrations being based in each instance on the weight of the active substance.

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*Photometric Turbidimetry.*—For the turbidimetric estimation of growth, broth cultures were incubated at 37° C. in optically matched cuvettes, and the readings were made at arbitrarily selected intervals in a Coleman Jr. spectrophotometer set at a wave length of 490 λ.* Fresh, fully grown cultures which had been incubated for sixteen to eighteen hours were used as the source of the inoculum. Dilutions of the cultures and of the antibiotics were all made in broth. Pour plates and colony counts were made in each instance to determine the size of the inoculum actually used, and occasionally also to follow the growth of the cultures. Cultures in antibiotic-free broth and an uninoculated broth blank were included each time. The per cent of light transmitted was read and recorded directly at the desired intervals, the instrument being calibrated so that the broth blank permitted either 100 or 90 per cent transmission of light, depending on the intensity of the color of the broth.

The sensitivity of a strain to any given antibiotic was defined on the basis of the changes in the per cent of light transmitted in the antibiotic-containing cultures as compared with that of the same culture in antibiotic-free medium. The "complete" and "partial" inhibiting concentrations were thus defined arbitrarily as the minimum concentrations of antibiotic which produced, respectively, less than one-tenth and less than one-half of the change observed in the per cent of light transmitted through the corresponding culture which contained no antibiotic.†

## RESULTS

With turbidimetric assays of antibiotics, it is theoretically possible to obtain highly accurate values fairly rapidly by proper choice of conditions for the test. In such assays all of the conditions are standardized except for the substance which contains the antibiotic to be assayed. The effect of the vehicle can also be minimized by selecting a highly sensitive organism for the assay; this usually permits considerable dilution of the material under assay to the point where it may have little or no effect, particularly if the diluent is the same as that used for the standard antibiotic solution with which the unknown is being compared.

In the case of the test for sensitivity, the growth of the organism in the medium may vary considerably from one strain to another with respect to the size of the inoculum required, the rate of growth, the time when maximum growth is achieved, the maximum density the culture will attain, the nature of the suspension it forms in the medium, the amount of lysis of organisms that may occur during growth, etc. Each strain must, therefore, be considered as a separate entity with respect to this test, and the effect of the various factors must be investigated in each instance. In addition, some of the antibiotics, in the concentrations required to inhibit growth of certain less sensitive strains, make solutions which are deeply colored and which themselves produce significant changes in color or turbidity during the course of incubation.

In the present study it was possible to consider only the more important of these factors, particularly the effect of the size of the inoculum and the duration of incubation. It also proved necessary to use several twofold, or occasionally fourfold concentrations of antibiotic with each organism in order to demonstrate both partial and complete inhibition under the chosen conditions, and in order to illustrate the nature of the turbidimetric growth curves when the test was

*At this wave length turbidity resulting from bacterial growth permitted a minimum of light transmission. The turbidity produced by the cultures that were studied permitted all observations, except during the earliest hours of incubation, to be made where the instrument error was the smallest, viz. less than 2 per cent. In observations where the changes in turbidity still allowed 80 per cent or more of light transmission, the instrument and reading errors amounted to 5 to 15 per cent of the observed changes.

†A similarly arbitrary definition could be based on the values for density (D), which can be derived from the per cent of light transmission (T), since $D = 2 - \log T$.

applied to different organisms and antibiotics. The features which were kept constant throughout the study were: (1) the antibiotics, the same lot of each agent being used throughout; (2) the culture medium, except insofar as the intensity of the color of batches varied, even though they were made in the same manner from the same lot of medium; (3) the electrophotometer, which during much of the study was supplied with power through an electronic voltage regulator and which was operated at a constant wave length of 490 λ; and (4) the time of incubation of the seed cultures which were grown at 37° C. for sixteen to eighteen hours.

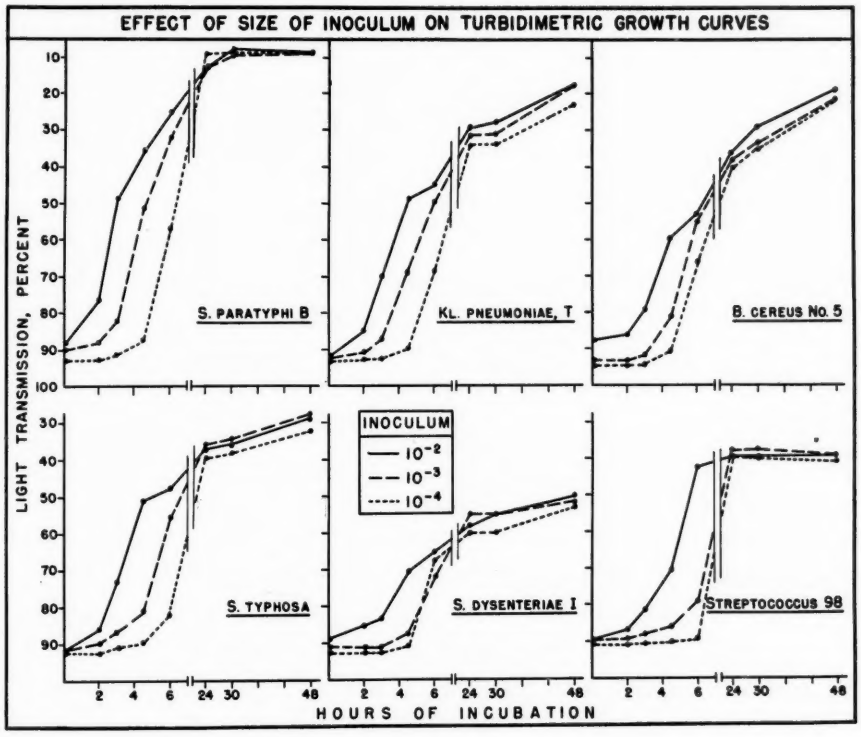


Fig. 1.

*Turbidimetric Growth Curves in Antibiotic-Free Medium: Effect of Size of the Inoculum.*—One of the most desirable features of turbidimetric methods for assay of antibiotics is that the conditions can usually be fixed so that the period of incubation is a brief one. This necessitates the use of a relatively large inoculum and the choice of a test organism with a rapid rate of growth so that there is a large, or even maximum increase in turbidity during the brief and fixed period of incubation. It is usually desirable and convenient to have a growth period of about six hours or less; this permits the test to be set up and read during the same work day. When this is not possible, it is preferable to have an optimum period for reading after eighteen to twenty-four hours which permits termination of the test on the following day. Since different organisms

vary in their rate of growth in the same medium, it was first desired to explore this aspect in order to determine whether a brief assay period was regularly feasible.

For this purpose, turbidimetric growth curves were made with each of the test organisms. The inocula used provided a final dilution of $10^{-2}$, $10^{-3}$, and $10^{-4}$ of the seed culture in a volume of 5 ml. of broth. Readings were made three or four times during the first six hours, and then at longer intervals between twenty-four and forty-eight hours. The results for six of the organisms are shown in Fig. 1. The growth curves for Staphylococcus 3 are not shown, but were essentially similar to those shown here for *S. typhosa.*

These growth curves illustrate the variability among different organisms in the length of the lag phase, in the rate at which the turbidity increases, in the maximum increase in turbidity that is attained, and in the time required to attain this maximum. The effect of the size of the inoculum on these aspects of the growth curves was qualitatively similar for the different organisms, but the net effect with respect to the applicability of such growth curves for rapid determination of sensitivity of the organisms was not the same in each instance. Thus the major part of the logarithmic phase of growth, as represented by the decrease in light transmission, occurred in the first six hours in most of the cultures in which a $10^{-2}$ inoculum was used. On the other hand, when a $10^{-4}$ inoculum was employed, some of the organisms, notably *S. typhosa, Streptococcus* 98, and *Staphylococcus* 3, showed little or no increase in turbidity at six hours. With the $10^{-3}$ inoculum, the growth was intermediate.

The increases in turbidity which occurred at earlier periods also varied markedly with the different organisms, but for most of them the decrease in the light transmitted was not adequate for the purpose at hand. After twenty-four hours or longer, the maximum decrease in light transmission was usually reached by all of the organisms and from each of the inocula used, although further and significant increases in turbidity were registered during the second twenty-four hour period in the case of *B. cereus* No. 5, and may have been due to clumping of the organisms. Slight increases in turbidity after twenty-four hours may also result from accumulation of products of growth, as in the case of *Kl. pneumoniae.*

From these observations it appears that, under the conditions selected, the optimum inoculum for the turbidimetric test should be $10^{-2}$, and that even with this large inoculum a minimum of six hours of growth may be required if the tests are to be applied to the variety of organisms that were used here.

*Variations in Growth of Organisms as Determined Turbidimetrically.*—Although the batch of dehydrated broth and the method of its preparation for use was the same throughout this study, the color of the broth was not constant after the medium was made up and sterilized at different times. The same was true for the density of the cultures used for the inoculum; these had been grown for sixteen to eighteen hours before use. Table I shows the variations observed in the growth of each of the cultures at different times, using a constant inoculum of 1 per cent in the absence of antibiotics.

The decrease in light transmission through the culture medium resulting from the addition of the inoculum varied from the least measurable amount to

about 10 per cent. There was usually only a slight increase in density after two hours of incubation, but occasionally there was enough growth during this period to decrease the light transmitted by more than 10 per cent. The variations were greatest for the individual cultures at four hours, and were the least marked at twenty-four hours. The final density attained also varied considerably both for each of the organisms at different times and for the different organisms.

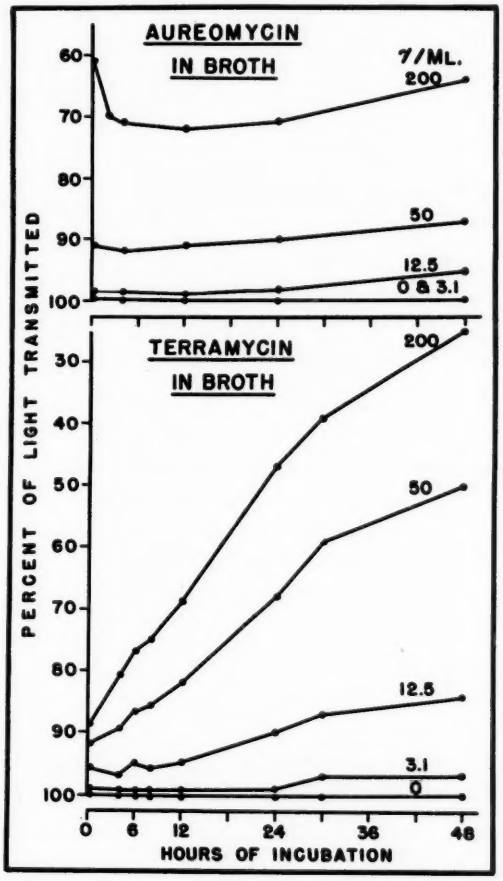


Fig. 2.—Effect of incubation of aureomycin and terramycin in broth on transmission of light at 490λ.

*Changes Produced by Incubation of the Antibiotics in Broth.*—The possibility that incubation of the antibiotics in broth may result in changes in light transmission due to changes either in solubility or in intensity of color was also considered. In the concentrations used in these studies, which ranged up to 200 micrograms per milliliter, only aureomycin and terramycin produced significant changes during the course of incubation. These are shown graphically in Fig. 2. In the case of both of these agents, increases in concentration resulted initially in increases in intensity of color and, in the case of aureomycin, possibly also

TABLE I. VARIATIONS IN GROWTH OF CONTROL CULTURES CONTAINING NO ANTIBIOTIC AS OBSERVED TURBIDIMETRICALLY

| ORGANISM | PER CENT OF LIGHT TRANSMITTED BEFORE INCUBATION (INOCULUM = $10^{-2}$) | REDUCTION IN PER CENT OF LIGHT TRANSMITTED* | | | | PER CENT OF LIGHT TRANSMITTED AFTER GROWTH FOR 24 HOURS |
|---|---|---|---|---|---|---|
| | | 2 HOURS | 4 HOURS | 6 HOURS | 24 HOURS | |
| *Salmonella typhosa* | 81-90 | 2-4 | 11-34 | 36-45 | 45-52 | 37-43 |
| *S. paratyphi*, B | 81-99 | 2-23 | 19-58 | 51-69 | 62-86 | 11-28 |
| *Shigella dysenteriae I* | 88-100 | 0-5 | 4-14 | 16-31 | 33-47 | 49-57 |
| *Klebsiella pneumoniae*, T | 87-100 | 2-12 | 17-41 | 44-59 | 52-66 | 28-40 |
| *Bacillus cereus*, No. 5 | 80-98 | 0-4 | 16-26 | 33-46 | 50-72 | 19-40 |
| *Staphylococcus aureus*, No. 3 | 89-99 | 0-9 | 2-36 | 7-67 | 52-75 | 23-37 |
| *Streptococcus*, 98 | 80-88 | 1-6 | 2-45 | 9-54 | 44-54 | 35-44 |

All observations were made in Coleman Junior Spectrophotometer at 490λ and are compared with uninoculated broth which was usually set as 100 per cent standard, but occasionally the broth standard was set at 90 per cent if it had more color than usual. The variations recorded are each based on from five to eight separate observations.

*Difference between per cent of light transmitted before incubation and at the designated hour.

slight turbidity due to failure to attain complete solution promptly. As a result there was a progressive decrease in the per cent of light transmitted before incubation was started.

As the solutions were incubated, the two antibiotics behaved differently. The higher concentrations of aureomycin showed an increase in the percent of light transmitted over a period of four hours, or longer. This was interpreted as possibly due, in part, to increased solution of the agent or possibly to changes in color. After twelve hours, the solutions all remained clear, but the intensity of the color increased in the broth which contained 12.5 γ or more per milliliter. This resulted in a decrease in the per cent of light transmitted, which was appreciable only during the second twenty-four hours of incubation, and even then was not marked.

In the case of terramycin there was a steady darkening of the solution with increase in intensity of color during incubation. However, the solutions remained clear, the changes were proportional to the concentration of antibiotic, progressed steadily during the forty-eight hour period of observation, and were associated with corresponding decreases in the per cent of light transmitted at the constant wave length used. In the tube containing 3.1 γ per milliliter, the change was not marked and was noted only after thirty hours or longer.

The decrease in transmission of light during incubation of terramycin solutions was considered to be due to chemical changes in the antibiotic; they have been observed in aqueous solutions of this agent on standing. Quantitatively the decrease in per cent of light transmitted through solutions containing 50 γ or more per milliliter of broth was of the same order of magnitude as that resulting from the increase in turbidity during growth of most of the cultures used in this study.

*Turbidimetric Growth Curves.*—Selected series of growth curves of organisms, starting with a 1 per cent ($10^{-2}$) inoculum in graded concentrations of four

antibiotics are shown in Fig. 3. These curves illustrate two patterns of growth as reflected in changes in light transmission. The first of these is exemplified by the growth of *Kl. pneumoniae* in the presence of terramycin, and by that of *S. typhosa* in chloramphenicol as shown in the upper panels of Fig. 3. The effect of increasing the concentration of the antibiotic in these instances appears to be one of progressively decreasing the rate of multiplication. The final density achieved, even after twenty-four or forty-eight hours of growth, was proportional to the concentration. The second pattern is illustrated by the

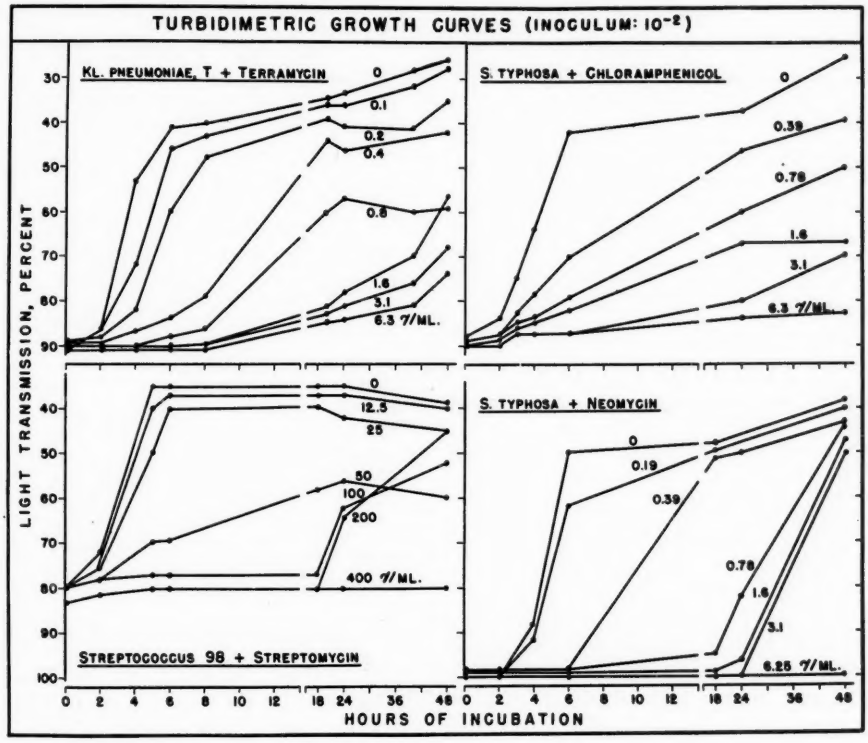


Fig. 3.

growth of *Streptococcus* 98 and *S. typhosa* in graded concentrations of streptomycin and neomycin, respectively, as shown in the lower panels of Fig. 3. In these instances, the effect of increasing the concentration of antibiotic was to increase progressively the lag phase of growth, but after that phase had passed the rate of growth and the final density attained by the cultures appeared to approach that of the control in which there was no antibiotic. Other examples are shown in Figs. 4, 5, and 6.

In the case of the antibiotics used in this study, only chloramphenicol and terramycin affected the growth curves in the manner indicated by the first pattern, and this was true for all the seven organisms studied. The second pattern of growth was produced by these same organisms when grown in either

TABLE II. SENSITIVITY OF 7 ORGANISMS TO 5 ANTIBIOTICS AS DETERMINED AT DIFFERENT STAGES OF GROWTH BY A TURBIDIMETRIC METHOD

| ORGANISM | HOURS OF INCUBATION | PENICILLIN | | STREPTOMYCIN | | AUREOMYCIN | | TERRAMYCIN | | CHLORAMPHENICOL | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C* | P† | C | P | C | P | C | P | C | P |
| *Salmonella typhosa* | 4 | -- | -- | 25 | 25 | 0.4 | 0.05 | 0.8 | 0.1 | 3.1 | 0.4 |
| | 6 | 3.1 | 1.6 | 25 | 25 | 0.4 | 0.1 | 0.8 | 0.2 | 3.1 | 0.8 |
| | 24 | 12.5 | 1.6 | >25 | >25 | 3.1 | 3.1 | 1.6‡ | 0.4 | 6.3 | 1.6 |
| *S. paratyphi*, B | 4 | 25 | 12.5 | 100 | 50 | 0.2 | 0.1 | <0.2 | <0.2 | 1.6 | 0.4 |
| | 6 | 12.5 | 12.5 | 100 | 100 | 0.4 | 0.2 | <0.2 | <0.2 | 1.6 | 0.8 |
| | 24 | 50 | 3.1 | >800 | >800 | 3.1 | 3.1 | 6.3‡ | 0.8 | 6.3 | 3.1 |
| *Shigella dysenteriae* I | 6§ | 6.3 | 3.1 | 25 | 12.5 | 0.4 | 0.05 | 0.4 | 0.2 | 1.6 | 0.4 |
| | 24 | 12.5 | 12.5 | 25 | 25 | >1.6 | 1.6 | 1.6‡ | 1.6 | 3.1 | 1.6 |
| *Klebsiella pneumoniae*, T | 4 | 50 | 12.5 | 3.1 | 0.2 | 0.2 | 0.05 | 0.8 | 0.1 | 0.8 | 0.4 |
| | 6 | 50 | 12.5 | 12.5 | 3.1 | 0.2 | 0.2 | 0.8 | 0.4 | 1.6 | 0.8 |
| | 24 | >800 | >800 | 12.5 | 12.5 | 12.5 | 3.1 | 3.1‡ | 1.6 | 3.1 | 0.8 |
| *Bacillus cereus*, No. 5 | 4 | 0.8 | 0.05 | 50 | 25 | 0.003 | 0.001 | 0.05 | 0.02 | 1.6 | 0.4 |
| | 6 | 3.1 | 0.2 | 50 | 25 | 0.01 | 0.003 | 0.05 | 0.02 | 1.6 | 0.8 |
| | 24 | 200 | 200 | 100 | 50 | 0.2 | 0.2 | 0.1‡ | 0.1 | 3.1 | 3.1 |
| *Staphylococcus aureus*, No. 3 | 4 | 3.1 | 0.8 | 12.5 | 3.1 | <0.1 | <0.1 | 0.05 | 0.05 | 6.3 | 1.6 |
| | 6 | 12.5 | 3.1 | 12.5 | 12.5 | <0.1 | <0.1 | 0.2 | 0.05 | 25.0 | 3.1 |
| | 24 | >200 | 200 | 50 | 50 | 0.2 | 0.2 | 0.8 | 0.8 | 25.0 | 12.5 |
| *Streptococcus* 98 | 6§ | 0.02 | 0.01 | 100 | 50 | <0.05 | <0.05 | <0.1 | <0.1 | 1.6 | 0.8 |
| | 24 | 0.02 | 0.02 | 400 | 100 | 0.8 | 0.8 | 0.4‡ | 0.4 | 3.1 | 1.6 |

Inoculum = $10^{-2}$ (1 per cent) in each instance.

*C = minimum complete inhibiting concentration in μg/ml, or less than 1/10 of reduction in per cent of light transmission observed at the same interval in the control culture containing no antibiotic.

†P = minimum partial inhibiting concentration in μg/ml defined as one-half or less of the reduction in per cent of light transmission observed at the same interval in control culture containing no antibiotic.

‡This and greater concentrations showed slight decreases in light transmission which were somewhat more than 1/10 of the increases observed in the controls but were considered to be due to changes in color rather than turbidity.

§In the case of these organisms growth at four hours was inadequate in the absence of antibiotic.

penicillin, streptomycin, or aureomycin, and also when neomycin was added to cultures of *S. typhosa* and *Sh. dysenteriae,* the only two organisms tested with that antibiotic.

The interpretation of these growth curves in terms of sensitivity as defined in this study is shown in Table II. The values for the six and twenty-four hour readings are given for all of the organisms, but those at four hours are indicated only for the organisms which achieved sufficient density in the control culture

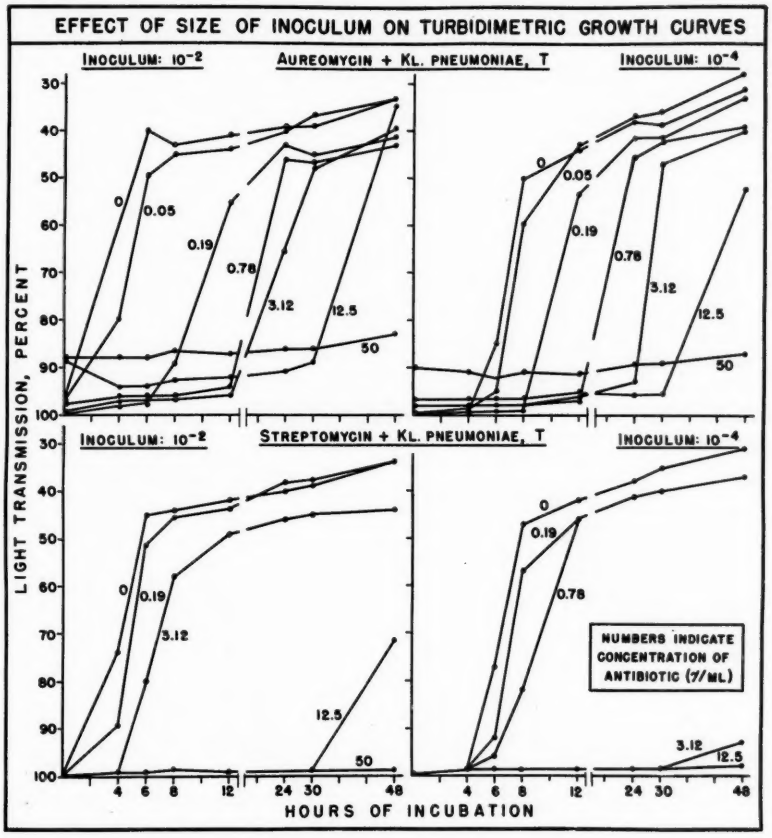


Fig. 4.

to permit the necessary comparisons at that interval. It is at once apparent that there were wide discrepancies in these sensitivity values depending on the period of incubation after which the readings were made. This was true with respect to each of the organisms and antibiotics, and irrespective of whether complete or partial inhibiting concentrations are considered. The discrepancies were somewhat greater, however, when the partial inhibiting concentrations were compared at different times. This was shown most strikingly with most organisms and aureomycin, and in the case of some organisms (*Kl. pneumoniae,* T, *B. cereus* 5 and *Staphylococcus* 3) was also marked with penicillin. The part played by the deterioration of aureomycin or, perhaps, of other antibiotics dur-

ing incubation was not studied. The ratio of complete to partial inhibiting concentrations, which may be a measure of the sharpness of the end point of the test was generally, but not always, smallest after the twenty-four hour interval.

*Effect of Size of Inoculum on Turbidimetric Growth Curves in Antibiotic-Containing Medium.*—It was previously shown in the turbidimetric representation of growth of cultures in antibiotic-free medium that the effect of progressively decreasing the size of the inoculum from $10^{-2}$ to $10^{-4}$ was essentially to

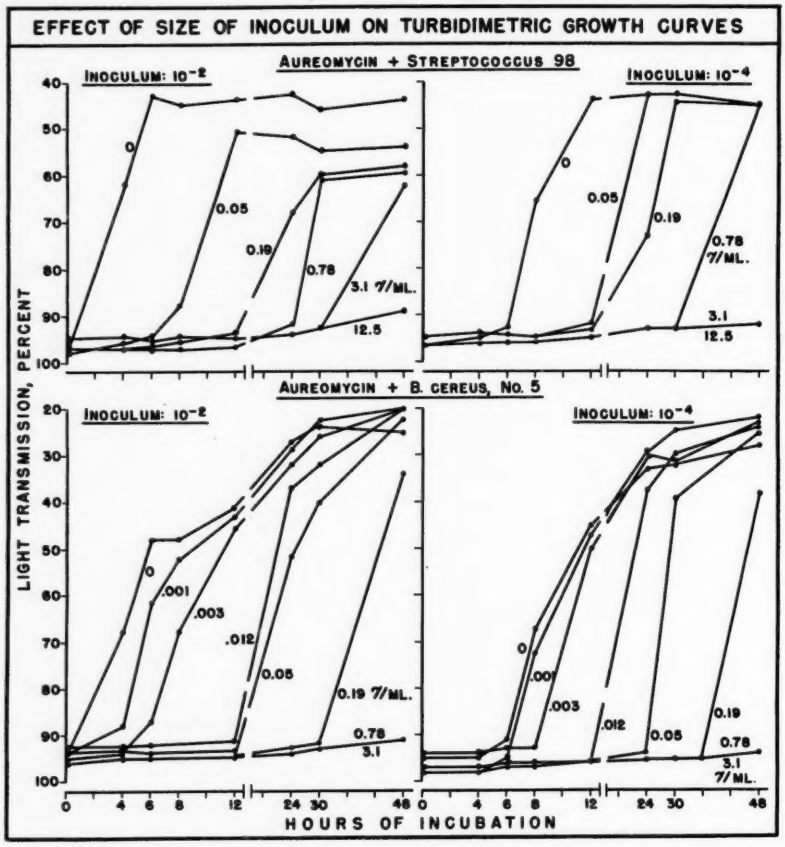


Fig. 5.

increase the lag phase of growth, as shown in Fig. 1. Once the lag phase was passed, the rate of decrease in the per cent of light transmitted and the final density attained by the cultures were essentially the same for each of the inocula used. In order to determine by this method the effect of the size of the inoculum on the sensitivity of organisms to antibiotics, parallel tests were set up simultaneously with inocula of $10^{-2}$ and $10^{-4}$ obtained from the same seed culture. Turbidimetric growth curves were then made with two- or fourfold concentrations of antibiotics. The resulting growth curves of *Kl. pneumoniae*, T in the presence of aureomycin and streptomycin are shown in Fig. 4.

As already indicated from the previous observations, increases in the concentrations of these antibiotics resulted in progressive increases in the period of incubation required before rapid growth began, as reflected in rapid decreases in the light transmitted through the cultures. The effect of decreasing the size of the inoculum appears to be that of further delaying the beginning of the logarithmic phase of growth. In the case of aureomycin, the final patterns of growth and the total amounts of growth, as reflected in the transmission of light, were

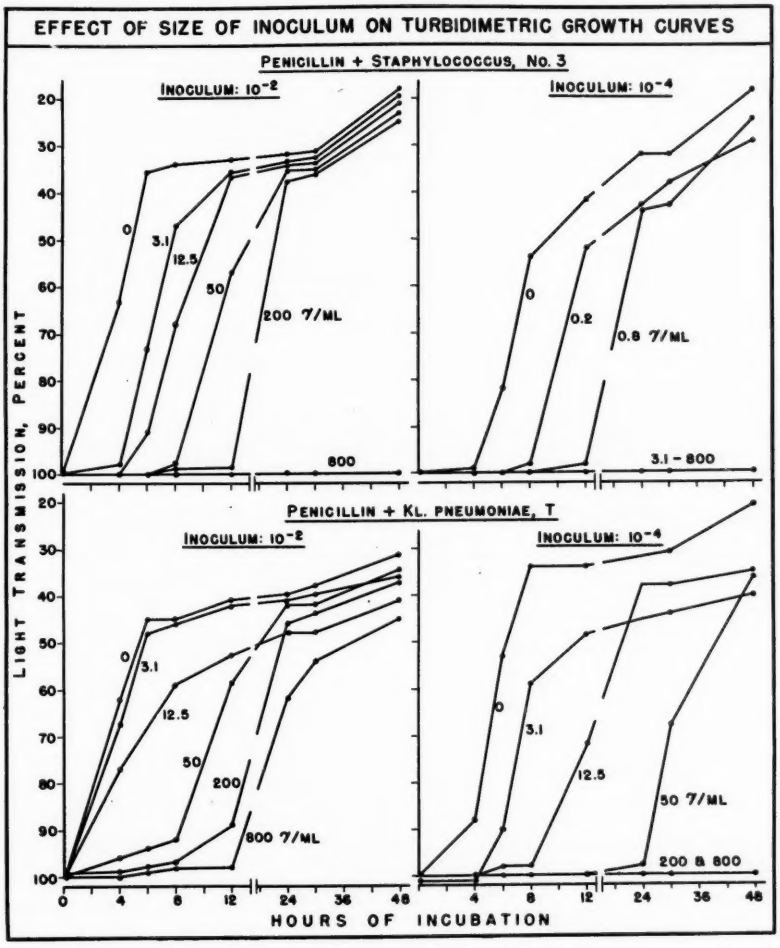


Fig. 6.

the same with each of the concentrations of the antibiotic and for both sizes of inoculum. With streptomycin, however, the use of the smaller inoculum resulted essentially in increasing the sensitivity of the organism so that a smaller concentration of the antibiotic was required to prevent any significant decreases in transmission of light through the culture.

The aureomycin effect was duplicated with *Streptococcus* 98 and with *B. cereus* No. 5, as shown in Fig. 5. An effect similar to that which occurred with

TABLE III. EFFECT OF SIZE OF INOCULUM ON SENSITIVITY OF ORGANISMS AS DETERMINED BY A TURBIDIMETRIC METHOD AFTER DIFFERENT PERIODS OF INCUBATION

| ORGANISM | ANTIBIOTIC | HOURS OF INCUBATION | MINIMUM INHIBITING CONCENTRATION, γ/ML. | | | |
|---|---|---|---|---|---|---|
| | | | INOCULUM $10^{-2}$ | | INOCULUM $10^{-4}$ | |
| | | | COMPLETE* | PARTIAL† | COMPLETE | PARTIAL |
| *B. cereus*, No. 5 | Aureomycin | 4 | 0.003 | 0.001 | ---‡ | --- |
| | | 6 | 0.012 | 0.003 | --- | --- |
| | | 8 | 0.012 | 0.012 | 0.003 | 0.003 |
| | | 12 | 0.012 | 0.012 | 0.012 | 0.012 |
| | | 24 | 0.19 | 0.19 | 0.05 | 0.05 |
| | | 30 | 0.19 | 0.19 | 0.19 | 0.19 |
| | | 48 | 0.78 | 0.78 | 0.78 | 0.78 |
| *Streptococcus* 98 | Aureomycin | 4 | 0.05 | <0.05 | <0.05 | <0.05 |
| | | 6 | 0.05 | <0.05 | <0.05 | <0.05 |
| | | 8 | 0.19 | 0.05 | <0.05 | <0.05 |
| | | 12 | 0.19 | 0.19 | <0.05 | <0.05 |
| | | 24 | 0.78 | 0.19 | 0.78 | 0.19 |
| | | 30 | 3.1 | 3.1 | 0.78 | 0.78 |
| | | 48 | 12.5 | 12.5 | 3.1 | 3.1 |
| *Kl. pneumoniae*, T | Aureomycin | 4 | 0.19 | 0.05 | --- | --- |
| | | 6 | 0.19 | 0.19 | 0.19 | 0.05 |
| | | 8 | 0.78 | 0.19 | 0.19 | 0.19 |
| | | 12 | 0.78 | 0.78 | 0.78 | 0.78 |
| | | 24 | 12.5 | 3.1 | 3.1 | 3.1 |
| | | 30 | 12.5 | 12.5 | 12.5 | 12.5 |
| | | 48 | 50. | 50. | 50. | 50. |
| *Kl. pneumoniae*, T | Streptomycin | 4 | 3.1 | 0.19 | --- | --- |
| | | 6 | 12.5 | 3.1 | 0.78 | 0.05 |
| | | 8 | 12.5 | 12.5 | 3.1 | 0.78 |
| | | 12 | 12.5 | 12.5 | 3.1 | 3.1 |
| | | 24 | 12.5 | 12.5 | 3.1 | 3.1 |
| | | 30 | 12.5 | 12.5 | 3.1 | 3.1 |
| | | 48 | 50. | 12.5 | 12.5 | 3.1 |
| *Streptococcus* 98 | Penicillin | 4 | 0.012 | 0.012 | --- | --- |
| | | 6 | 0.012 | 0.012 | --- | --- |
| | | 8 | 0.012 | 0.012 | 0.012 | 0.012 |
| | | 12 | 0.012 | 0.012 | 0.012 | 0.012 |
| | | 24 | 0.012 | 0.012 | 0.012 | 0.012 |
| | | 30 | 0.012 | 0.012 | 0.012 | 0.012 |
| | | 48 | 0.012 | 0.012 | 0.012 | 0.012 |
| *Staphylococcus*, No. 3 | Penicillin | 4 | 0.78 | 0.78 | --- | --- |
| | | 6 | 12.5 | 0.78 | <0.19 | <0.19 |
| | | 8 | 50. | 12.5 | <0.19 | <0.19 |
| | | 12 | 200. | 200. | 0.78 | 0.78 |
| | | 24 | 800. | 800. | 3.1 | 3.1 |
| | | 30 | 800. | 800. | 3.1 | 3.1 |
| | | 48 | 800. | 800. | 3.1 | 3.1 |
| *B. cereus*, No. 5 | Penicillin | 4 | 3.1 | 0.19 | --- | --- |
| | | 6 | 12.5 | 3.1 | 0.19 | <0.19 |
| | | 8 | 12.5 | 12.5 | 0.78 | 0.19 |
| | | 12 | 50. | 50. | 3.1 | 3.1 |
| | | 24 | 200. | 200. | 3.1 | 3.1 |
| | | 30 | >200. | >200. | 3.1 | 3.1 |
| | | 48 | >200. | >200. | 3.1 | 3.1 |
| *Kl. pneumoniae*, T | Penicillin | 4 | 50. | 12.5 | <0.78 | <0.78 |
| | | 6 | 50. | 12.5 | 12.5 | <0.78 |
| | | 8 | 200. | 50. | 12.5 | 12.5 |
| | | 12 | 800. | 200. | 50. | 12.5 |
| | | 24 | >800. | >800. | 50. | 50. |
| | | 30 | >800. | >800. | 200. | 50. |
| | | 48 | >800. | >800. | 200. | 200. |

TABLE III—CONT'D

| ORGANISM | ANTIBIOTIC | HOURS OF INCUBATION | MINIMUM INHIBITING CONCENTRATION, γ/ML. | | | |
|---|---|---|---|---|---|---|
| | | | INOCULUM $10^{-2}$ | | INOCULUM $10^{-4}$ | |
| | | | COMPLETE* | PARTIAL† | COMPLETE | PARTIAL |
| *Kl. pneumoniae*, T | Chloramphenicol | 4 | 0.8 | 0.2 | --- | --- |
| | | 6 | 0.8 | 0.8 | 0.8 | 0.2 |
| | | 8 | 3.1 | 0.8 | 0.8 | 0.8 |
| | | 12 | 3.1 | 0.8 | 0.8 | 0.8 |
| | | 24 | 3.1 | 0.8 | 3.1 | 0.8 |
| | | 30 | 3.1 | 0.8 | 3.1 | 0.8 |
| | | 48 | 3.1 | 0.8 | 3.1 | 0.8 |
| *Staphylococcus*, No. 3 | Terramycin | 4 | 0.05 | 0.05 | --- | --- |
| | | 6 | 0.2 | 0.05 | 0.05 | 0.01 |
| | | 8 | 0.2 | 0.05 | 0.05 | 0.05 |
| | | 12 | 0.2 | 0.2 | 0.2 | 0.05 |
| | | 24 | 0.2 | 0.2 | 0.2 | 0.2 |
| | | 30 | 0.2 | 0.2 | 0.2 | 0.2 |
| | | 48 | 0.8 | 0.2 | 0.8 | 0.8 |

*Reduction in per cent of light transmitted equal to $\frac{1}{10}$ or less of that observed in control.
†Reduction in per cent of light transmitted equal to $\frac{1}{2}$ or less of that observed in control.
‡- - - Insufficient growth in control culture.

streptomycin was illustrated even more clearly when penicillin was used in tests on *Staphylococcus* No. 3 and on *Kl. pneumoniae*, T. These curves are shown in Fig. 6.

The interpretations of these turbidimetric growth curves in terms of the sensitivity of the organisms as determined at different periods of incubation are shown in Table III. This table includes all of the combinations of organisms and antibiotics that are illustrated in Figs. 4, 5, and 6, and also four additional combinations. On the whole, the greatest differences between the results obtained with inocula of $10^{-2}$ and $10^{-4}$ were noted after the shorter periods of incubation. These differences, however, were observed only with penicillin, streptomycin, and aureomycin. In this method, the size of the inoculum had no significant effect on the sensitivity to chloramphenicol and terramycin which were used in the tests with *Kl. pneumoniae*, T and *Staphylococcus* No. 3, respectively.

Aureomycin also differed from penicillin rather strikingly. The former yielded similar end points with each of the two inoculum sizes after incubation for twenty-four hours or more, and sometimes even after eight or twelve hours. Except in the case of *Streptococcus* 98, the concentrations of penicillin required to produce the same degree of inhibited growth were usually much greater when the larger inoculum was used, and this was true for both the shorter and the longer periods of incubation.

It is also of interest to compare the results obtained with the $10^{-2}$ inoculum, as shown in Table III, with the results of corresponding tests and at the same intervals listed in Table II. These tests were done at different times, some of them several months apart. In the great majority of instances the values obtained were identical on both occasions, but a few showed differences equivalent to that of a single dilution, and hence were within the limits of the method. In the case of *Staphylococcus* No. 3 and penicillin, there was a very wide discrepancy in the readings at four hours, but this was much less marked at six

hours, and not significant at twenty-four hours. These discrepancies, especially in the early phase of growth and similar ones noted on other occasions in other tests, were associated with considerable differences in the rate of growth of the control cultures at different times. These and other findings suggest that the results of turbidimetric tests for sensitivity can be duplicated within limits of error similar to those of other methods which utilize serial dilutions of the order used here.

In summary, the size of the inoculum in the range employed here had a variable effect on the result of the turbidimetric test for sensitivity depending on the antibiotic used, the period of incubation, and the organism tested.

*Turbidity-Concentration Curves After Selected Periods of Incubation.*— The density of the culture, as measured by the per cent of light transmitted, could be plotted against the concentration of antibiotic for any organism after any desired period of growth. If this is done at successive intervals of growth, one may obtain a series of turbidity-concentration curves for the particular organism grown in any given antibiotic. This has been done for five combinations of organisms and antibiotics using a $10^{-2}$ inoculum in each instance. The resulting curves are shown in Fig. 7. These curves may be used for reading the sensitivity of the organism directly from the concentration scale. To do so, however, it is obviously necessary to define the sensitivity arbitrarily in terms of the time of incubation and the extent of inhibition one wishes to accept as the end point.

In Fig. 7, each of the curves is intersected by a short horizontal line at a level equal to one-half of the vertical distance between its starting point at the left (which represents the per cent of light transmitted by the control culture that contains no antibiotic) and the point of maximum light transmission on that curve (that is, where there is complete inhibition of growth). The reflection of any one of these points of intersection on the abscissa gives the closest approximation to the "partial inhibiting concentration" as defined in this study for the particular time interval represented by that curve. Any one of these values may be considered to be equivalent to what is usually referred to loosely as the "50 per cent inhibiting concentration."

It is at once apparent, however, that the value for the partial inhibiting concentration varies widely depending on which of the curves is chosen; that is, on the time when the test is read. Thus, for *Staphylococcus* No. 3 and chloramphenicol this value is between 0.8 and 1.6 $\gamma$ per ml. at four hours, between 1.6 and 3.1 $\gamma$ at six and eight hours, between 3.1 and 6.3 $\gamma$ at eight and twelve hours, and between 6.3 and 12.5 $\gamma$ per milliliter at thirty-four hours.

In a similar manner one may choose to define the sensitivity of an organism to any antibiotic as the minimum complete inhibiting concentration of the antibiotic. This would represent the point of maximum light transmission on that curve. From this point of view these groups of curves fall into two general patterns. In one instance the sensitivity would vary markedly with the time of incubation; this is illustrated best in the case of *S. typhosa* in neomycin in Fig. 7. In the second pattern which is illustrated in the case of *Sh. dysenteriae*

in streptomycin, the end point may be sharp and may be observed early; the value for sensitivity would thus be the same on each of the curves for subsequent periods of incubation. In the present study an allowance was made for increases in turbidity due to various factors, including the added drug, evaporation, and errors in the instrument. Hence, the complete inhibiting concentration was taken arbitrarily as the point where the curve reaches one-tenth or less of the vertical distance above the point of maximum light transmission.

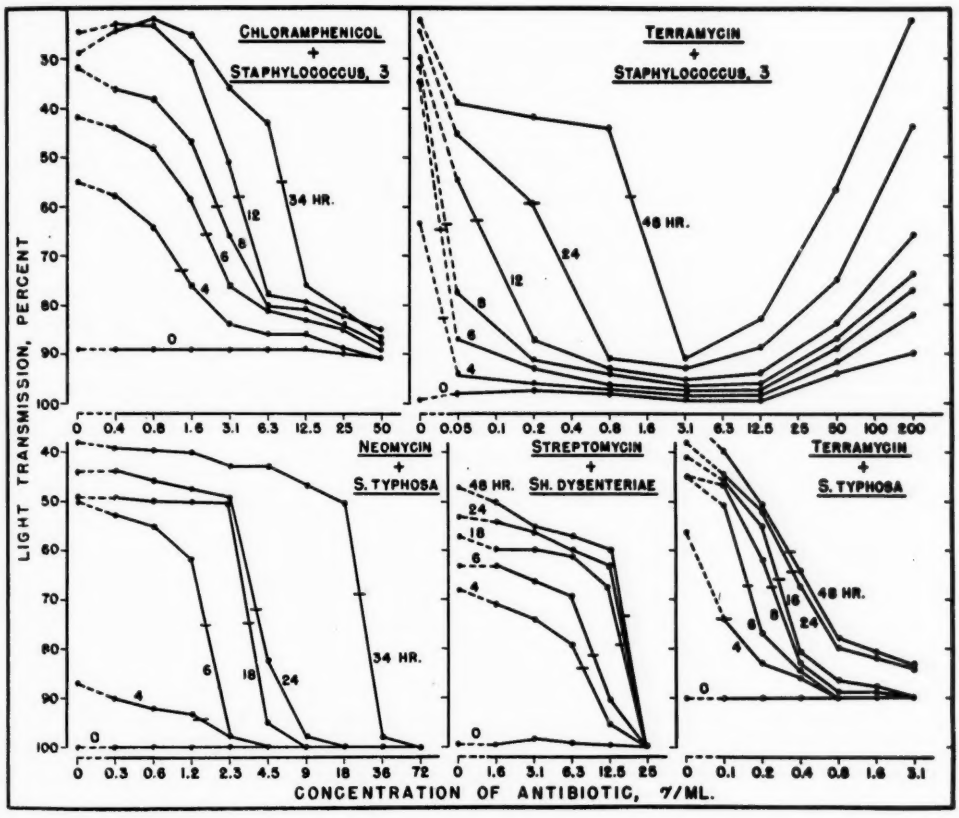


Fig. 7.—Turbidity-concentration curves at selected periods of incubation for five combinations of organisms and antibiotics.

The turbidity-concentration curves for the growth of *Staphylococcus* No. 3 in terramycin stand out as distinct from all of the others shown in Fig. 7. The curves representing different times of incubation follow the same pattern as those on the other panels in this figure until the concentration of 3.1 γ per milliliter is reached. At that point the maximum light is transmitted at each period of incubation. From there on, the per cent of light transmitted decreased progressively with increases in the concentration and in the time of incubation. These changes were associated with corresponding increases in the character and intensity of color of the antibiotic-containing medium and was not accompanied by any perceptible appearance of turbidity, even at the point where the

least amount of light was transmitted. A similar effect was noted in all tests in which these high concentrations of terramycin were used. In the case of the curves for *S. typhosa* in terramycin shown in Fig. 7, the portions of the curves beyond 3.1 $\gamma$ per milliliter have been omitted in order to conserve space on the chart. These changes correspond to the ones shown in Fig. 2, and represent the effect of incubation of terramycin in the absence of bacterial growth.

*Effect of Size of Inoculum on Turbidity-Concentration Curves.*—The curves shown in Fig. 7 were all obtained with the use of a $10^{-2}$ inoculum. Similar turbidity-concentration curves were also made for cultures grown simultaneously from $10^{-2}$ and $10^{-4}$ inocula obtained from the same seed culture. Such curves constructed for two combinations of organisms and antibiotics are shown in Fig. 8. These two combinations illustrate the two distinct types of effect which result from reducing the size of the inoculum. In the case of *Kl. pneumoniae* in aureomycin there was no significant effect from reducing the inoculum except that resulting from the sparser growth at eight hours or earlier. This early effect was also noted in the case of *B. cereus* grown in penicillin, but in this instance the smaller inoculum produced a sharp end point of inhibition in 3.1 $\gamma$ per milliliter at twelve hours and thereafter, whereas, with the $10^{-2}$ inoculum, progressively greater concentrations were required to produce the same effect as the period of incubation was increased.

The points on the curves corresponding to the "50 per cent inhibition" are also shown in Fig. 8, and the values for partial inhibiting concentrations of antibiotic can be read off on the concentration scale, as already indicated. The sensitivity of *Kl. pneumoniae* to aureomycin as determined in this manner at eight hours or at any one of the later intervals is almost identical for the two sizes of inoculum. When applied to the penicillin sensitivity of *B. cereus,* however, the two inocula yielded entirely divergent values. The values were much greater with the larger inoculum after any given period of incubation. Furthermore, the values for partial inhibiting concentrations increased progressively during prolonged incubation from the $10^{-2}$ inoculum, whereas with the inoculum of $10^{-4}$ values reached a sharp end point at about 1.6 $\gamma$ per milliliter at twelve hours, and did not increase significantly after that time.

The curves of *Kl. pneumoniae* in aureomycin also illustrate the effect of incubation of high concentrations of this antibiotic. The upswings in the parts of these curves beyond 3.1 $\gamma$ per milliliter were due to color changes and not turbidity, and correspond to the changes represented in Fig. 2.

## DISCUSSION

Some of the many factors which influence the results of tests for sensitivity of bacteria to antibiotics have already been referred to earlier in this paper. They have been reviewed more fully by Heatley.[2] Theoretically, turbidimetric methods employing photoelectric measurements offer greater precision with less effort than is required in most other quantitative methods when applied to the assay of antibiotics. Since such methods have been employed successfully for assay purposes, it seemed worth exploring their applicability to the testing of

the sensitivity of organisms to antibiotics. The data are presented only from the point of view of indicating the potentialities of one such method and not for the purpose of advocating its use.

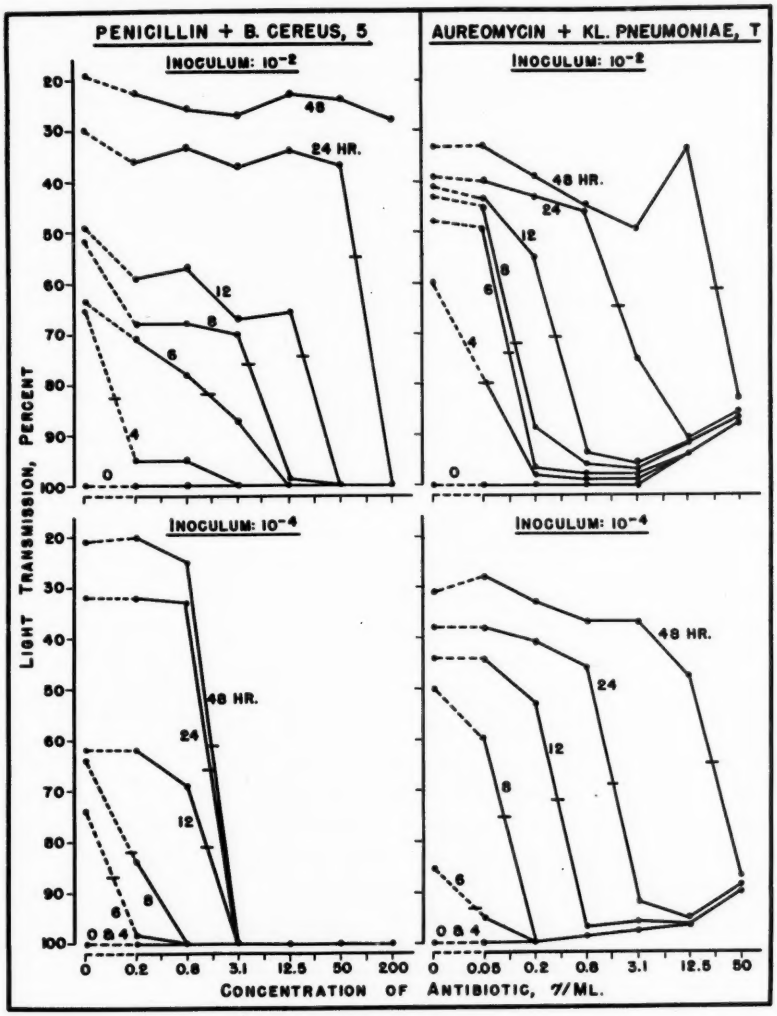


Fig. 8.—Effect of size of inoculum on turbidity-concentration curves after successive periods of growth.

Tests for sensitivity of organisms are used in screening antibiotics for their prospective therapeutic usefulness. For this purpose standard organisms with properties particularly suitable for the purpose or with known growth characteristics in specially selected media may be employed. Some of the inherent variations in the results of tests which depend on these characteristics may thus be minimized. The more important use of tests for sensitivity, however, is the choice of antibiotics for therapy or in the interpretation of the results of their use. That obviously implies the testing of organisms when they are first isolated,

or shortly thereafter when their exact growth patterns in any given medium cannot be predicted. Unavoidable sources of variations are thus introduced which must be taken into account in the interpretation of the results. This is equally true of the turbidimetric method as of others in general use because the action of the various antibiotics is dependent on many of these growth characteristics.

In the present study attention was focused primarily on two important factors and their effect on the results of the turbidimetric test; they are the size of the initial inoculum and the period of incubation. It was shown that each of these factors may influence the results of the test profoundly. Furthermore, the effect of each of these factors varies markedly depending both on the organism and on the antibiotic used for the test. The possibility of adapting such a method for rapid determination of sensitivity introduced the greatest source of variation due to the great differences among bacteria in the early phases of growth, depending on the size of the inoculum.

Assays for antibiotics, except those which depend on chemical quantitations, are, of course, subject to all of the variables involved in the tests for sensitivity. In the case of the assays, however, all of the factors except the amount of antibiotic in the substance under assay are kept constant. This makes possible reasonably reliable comparisons with the standard control material of known antibiotic content.

The data presented point up the extreme caution with which the results of tests for sensitivity of bacteria to antibiotics must be interpreted from the point of view of their applicability in clinical practice. Variations in the values obtained in the same test with the same combination of organism and antibiotic may be minimal, as in the case of *Streptococcus* 98 and penicillin, as shown in Table III. On the other hand, they may vary more than one thousand fold depending on the size of the inoculum used and on the time and endpoint chosen for reading the test as illustrated in the same table in the case of either *Staphylococcus* No. 3 or *Kl. pneumoniae*, T in the same antibiotic. The resulting difficulties in choosing agents for therapy and in correlating the results of such tests with the clinical effects of the agents are obvious.

A comparison of the results of tests obtained by this method and by other methods which are in more general use is presented separately elsewhere.[3]

## SUMMARY AND CONCLUSIONS

Turbidity, as measured electrophotometrically, was used to determine the sensitivity of a variety of strains of bacteria to several antibiotics. The minimum complete and partial inhibiting concentrations of these antibiotics were determined for each of the organisms according to arbitrarily defined standards after observing the inhibition of turbidity during growth in graded concentrations of antibiotics, as compared with similar cultures grown simultaneously in antibiotic-free medium.

The sensitivity determined in this manner was significantly altered by differences in the number of bacteria in the inoculum and by the length of the period of growth.

The effects of these factors also varied with the organisms and with the antibiotics that were tested.

Two distinct patterns of inhibition of bacterial growth, as reflected in the turbidimetric growth curves, were observed among the five antibiotics that were studied. There were also two types of effects that resulted from the reduction in the size of the inoculum. Each of these effects was reflected in marked differences in the values obtained with the sensitivity tests, as determined in this manner.

An appreciation of the effects of these factors is essential for the proper interpretation of the results of these tests.

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# HUMAN SERUM TRIBUTYRINASE

## I. Normal Human Serum Tributyrinase Levels

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A TITRIMETRIC micromethod was developed by Tuba and Hoare[1] for estimating the lipolytic activity of the serum of rats under a variety of experimental conditions. This simple method, which resembles the macromethod of Goldstein, Epstein, and Roe,[2] was modified in order to permit studies of the tributyrinase activity of human beings in health and in certain disease states including tuberculosis. Modifications were necessitated by the fact that human serum possesses a much lower lipolytic activity than rat serum. Essentially these consisted of utilization of larger amounts of serum and longer periods of lipolysis in order to insure the production of sufficient butyric acid to permit accurate titration.

Tributyrinase activity was estimated in the sera of 280 normal persons. This included subjects of both sexes, and ages ranging from 9 years to 63 years.

### MATERIALS AND METHODS

*Factors Affecting Tributyrinase Activity.*—Tuba and Hoare found emulsifiers to be unnecessary when tributyrin is used as a substrate. Vigorous mixing of the buffered serum with the substrate at the moment of addition and then mechanical agitation during the lipolytic period are sufficient to maintain tributyrin as an emulsion.

It was noted by Tuba and Hoare that calcium chloride increased rat tributyrinase activity 24 per cent. However, calcium activation was not adopted in studies with human serum in order to keep the technique as simple as possible.

The time-activity curve for human serum is shown in Fig. 1. The lipolytic period of sixty minutes chosen for our investigations is on the linear portion of the curve.

The relationship between enzyme concentration and tributyrinase activity is illustrated in Fig. 2. To varying amounts of serum 0.020 per milliliter substrate was added in order to obtain each point on the graph. It is apparent that this quantity of tributyrin is sufficient to saturate all the enzyme contained in 0.250 milliliter of serum, which was chosen as a satisfactory amount for the investigations.

The effect of pH is indicated in Fig. 3. The graph is obtained using the average of the pH values at the beginning and the termination of a one-hour lipolytic period with each of several veronal buffers. The optimum activity was obtained with a buffer of pH 8.6. The average pH of the experimental tube was 8.15 during lipolysis, and the actual magnitude of the pH change in one hour was 0.42 unit.

*Method.*—The reagents are identical with those used in the micromethod[1]:

*Substrate:* Tributyrin
*Buffer:* 0.1 M sodium diethylbarbiturate, adjusted with 0.1 M hydrochloric acid to pH 8.6
*Lipase inactivator:* 95 per cent ethyl alcohol
*Sodium hydroxide:* 0.025 N

From the Department of Biochemistry, University of Alberta.
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Micro-Kjeldahl tubes with 5 ml. capacity bulbs are used. For each determination a control and an experimental tube are required. The contents are identical except for the use of boiled serum in the control tube. Into the experimental tube are pipetted 0.250 ml. serum, 0.250 ml. water, and 1.00 ml. veronal buffer of pH 8.6. After this mixture has been warmed to 37° C., 0.020 ml. tributyrin, previously warmed to 37° C., is added and mixed vigorously by hand. The tubes are then agitated for sixty minutes on a Warburg shaker at the rate of one hundred twenty swings per minute, which is sufficient to maintain the substrate in a state of fine dispersion. Enzyme action is terminated, and the serum proteins are precipitated by the addition of 4.0 ml. of 95 per cent ethyl alcohol. After centrifuging, the supernatant is decanted into 25 ml. erlenmeyer flasks and titrated at once using ten drops 0.04 per cent phenolphthalein as an indicator, to a faint but permanent pink color.

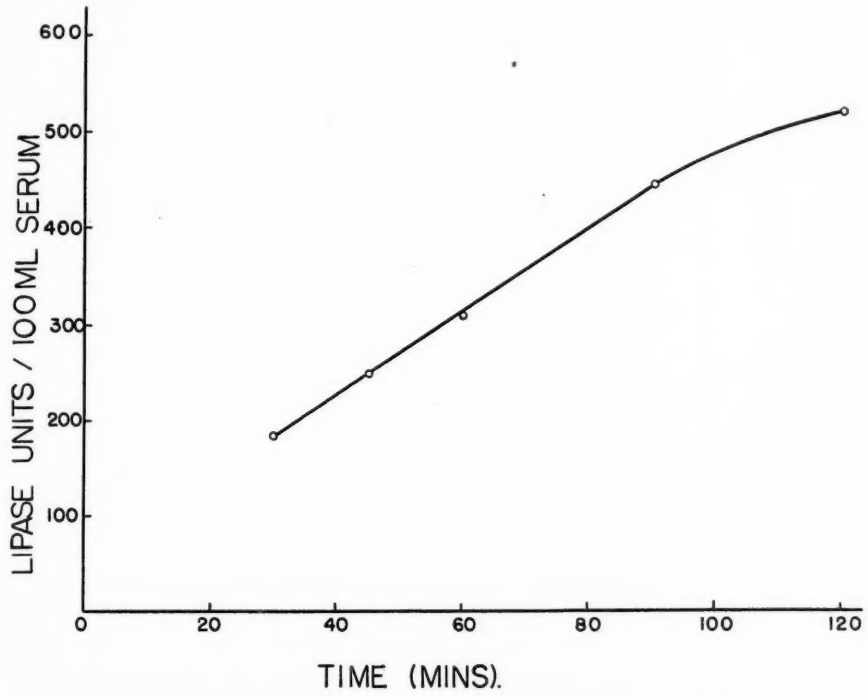


Fig. 1.—Relationship between time of hydrolysis and tributyrinase activity of human serum.

Tributyrinase activity is represented by the difference in titration between the contents of the experimental and control tubes. One milliliter of 0.025 N base is equivalent to one lipase unit. Serum lipase activity is reported on the basis of the number of milliliter of 0.025 N sodium hydroxide required to neutralize the butyric acid set free by 100 ml. of serum under the above conditions. A total of 0.5 ml. serum is sufficient for each determination of tributyrinase concentration. However, when a number of determinations are run at once, one control tube is usually found to be sufficient, and 0.25 ml. serum is adequate for some samples.

## RESULTS

With the aid of the simple semi-micro method just described, the tributyrinase activity of the sera of 280 children and adults was determined. It was felt that this would provide an adequate basis for comparison of the lipolytic activity of the sera of normal persons with those suffering from various disease

TABLE I. SERUM TRIBUTYRINASE LEVELS IN NORMAL SUBJECTS

| | NUMBER OF SUBJECTS | AVERAGE AGE (9-63 YEARS) | MEAN TRIBUTYRINASE ACTIVITY IN UNITS/100 ML. | STANDARD DEVIATION | STANDARD ERROR OF THE MEAN |
|---|---|---|---|---|---|
| All Subjects | 280 | 27 | 403 | ±89 | ±5.0 |
| Males | 174 | 29 | 417 | ±77 | ±5.8 |
| Females | 106 | 23 | 379 | ±75 | ±7.4 |
| Comparison of Means for males and females | | $t = 4.04$<br>$t$ at 1% level $= 2.60$ | | | |

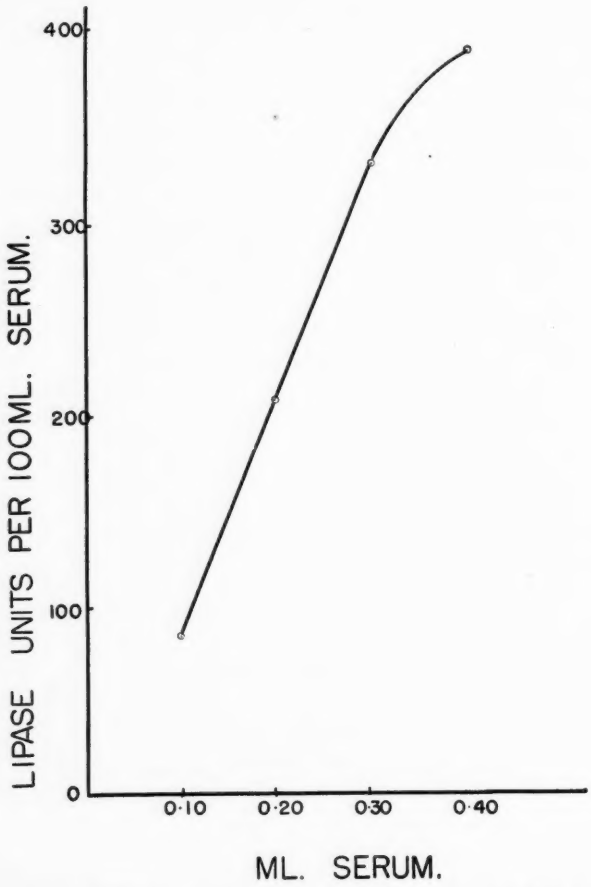


Fig. 2.—Relationship between enzyme concentration and tributyrinase activity.

states. It is apparent from Table I that the mean tributyrinase level of males is significantly higher than for females of the same age group, 9 to 63 years.

Because our studies would probably include children as well as adults, mean tributyrinase levels are arranged into various age groups in Table II.

Statistical comparisons of the youngest and the oldest with intermediate age groups indicate that there are significant changes with age in tributyrinase levels. The first group has been compared with the next four intermediate

TABLE II. SERUM TRIBUTYRINASE LEVELS AT DIFFERENT AGE INTERVALS IN NORMAL SUBJECTS

| AGE-INTERVAL (YEARS) | NUMBER OF SUBJECTS | MEAN TRIBUTYRINASE ACTIVITY IN UNITS/100 ML. | STANDARD DEVIATION | STANDARD ERROR OF THE MEAN |
|---|---|---|---|---|
| 9-14 | 59 | 444 | ±73 | ±9 |
| 15-19 | 48 | 397 | ±38 | ±6 |
| 20-24 | 33 | 362 | ±74 | ±13 |
| 25-29 | 23 | 379 | ±126 | ±26 |
| 30-34 | 29 | 377 | ±89 | ±17 |
| 35-44 | 54 | 394 | ±83 | ±11 |
| 45-63 | 32 | 442 | ±87 | ±15 |
| Totals | | | | |
| 9-63 years | 280 | 403 | ±89 | ±5.0 |

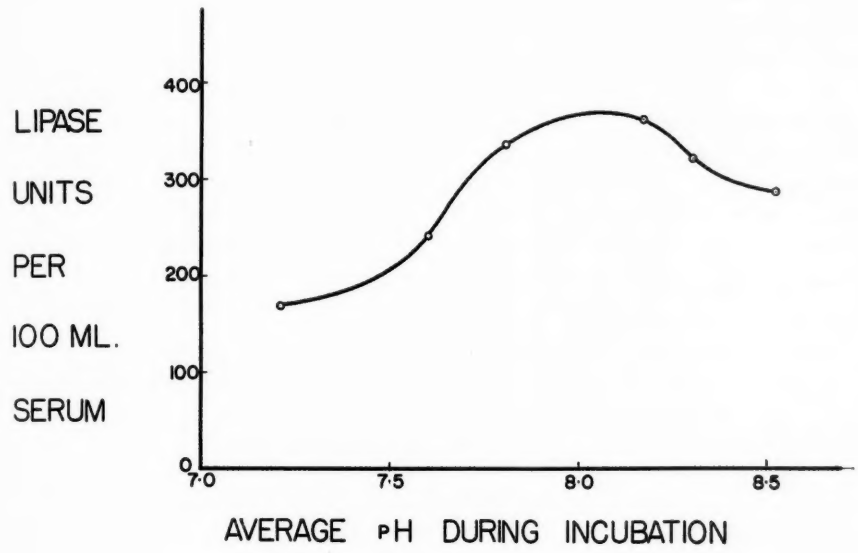


Fig. 3.—Effect of pH on human serum tributyrinase activity.

groups, and "t" at 1 per cent level has been found to be significantly different except in the 25 to 29 year group. A significant difference also exists between the oldest group 45 to 63 years, and an intermediate group, 30 to 34 years.

## SUMMARY

A simple titrimetric semi-micro method for the estimation of tributyrinase activity in human serum is described.

The normal range of activity from 9 to 63 years is 300 to 600 units per 100 milliliters. These values are higher than those usually reported in the literature, and for this reason it is felt that the subnormal variations occurring in certain disease states can be more accurately determined and followed over a period of time. This has proved valuable in subsequent determinations done in this laboratory.

The concentration of this enzyme in the sera of males is significantly higher than in that of females, as reported by Goldstein, Epstein, and Roe.[2] There are also significant variations of serum tributyrinase levels related to age.

The authors wish to thank Dr. H. Siemens for his assistance in obtaining some of the samples of blood serum.

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# QUANTITATIVE ESTIMATION OF THE RICKETTSIAL ANTIGENS IN INFECTED YOLK SAC MEMBRANES BY COMPLEMENT FIXATION USING A FIFTY PER CENT HEMOLYSIS TECHNIQUE

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STUDIES on the factors affecting the growth of pathogenic rickettsiae require some quantitative means of demonstrating the numbers of rickettsiae present in the material being examined. The quantitative methods available to research workers in this field involve embryonated hens' eggs, human body lice, guinea pigs, mice, cotton rats, or an in vitro hemolysin test for the demonstration of the presence of live rickettsiae. All of these methods are expensive and time consuming; consequently there is a need for an in vitro test which would give reproducible, specific results which could be used as an index of the numbers of rickettsiae, living and dead, in the medium being studied. The purpose of this paper is to report experiments adapting the Kent-Rein complement fixation methods[1] to the study of the antigenicity of yolk sac membranes infected with typhus rickettsiae. The results indicate that the technique as adapted herein is several times as sensitive for quantitative studies of total rickettsial content as the conventional complement fixation technique.

## MATERIALS AND METHODS

The demonstration of specific rickettsial antigen quantitatively in infected yolk sac membrane suspensions by complement fixation requires antiserum as free as possible from antibodies to yolk sac membrane components so that the results of the complement fixation test will not be masked by any interaction between yolk sac antigen and yolk sac antibody. Antirickettsial serum free from yolk sac antibody was produced for this work by immunizing rabbits against an antigen prepared from infected human body lice.[2]

*Preparation of Infected Louse Antigen.*—Under nembutal anesthesia a normal rabbit was given intravenously 6 ml. of 10 per cent *Rickettsia prowazeki* infected yolk sac membrane suspension and several pads of normal human body lice, *Pediculus humanus corporis,* allowed to feed on its abdomen according to the method of Snyder and Wheeler.[2] Samples of lice were taken daily and examined for the presence of rickettsiae in Macchiavello stained smears of their intestinal contents. All lice, approximately 200, remaining alive on the sixth day after the infecting meal were carefully harvested into a chilled Griffith tube, washed three times with nutrient broth and once with sucrose PG,[3] and then thoroughly ground in 18 ml. of sucrose PG. This suspension did not kill mice in the test for rickettsial toxin; a cotton rat immunity test indicated that there were approximately $10^{5.5}$ viable *R. prowazeki* per milliliter. The method by which this figure was obtained is similar to that of Snyder and associates.[4] This same procedure was followed for the preparation of a louse suspension infected with murine typhus; this was used to prepare antimurine rabbit serum.

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*Preparation of Antirickettsial Serum.*—An ampule of the infected louse antigen was thawed, reground in an insect grinding tube, and diluted 1 to 5 in sucrose PG, centrifuged at 800 r.p.m. for five minutes to remove any gross particles, and used for the immunization of rabbits. Three 1 ml. doses were given at five-day intervals. On the fourteenth day post inoculation the rabbit sera were tested for antibody content with celite washed rickettsial antigen of known titer. Antibody titers ranged from 1:160 to 1:1280. The sera were stored without preservatives at -10° C.

*Preparation of Infected Yolk Sac Membrane Antigen.*—Antigens for use in complement fixation work should be as free as possible from nonspecific and anticomplementary substances. Antigens in this work were prepared according to the method of Topping and Shepard.[6] The 50 per cent yolk sac membrane suspension was diluted 1 to 5 with the barbiturate buffered saline[7] used as diluent in the complement fixation procedure. It was extracted with an equal volume of diethyl ether, the aqueous layer freed from residual ether in a desiccator over concentrated sulfuric acid at room temperature, and used as antigen in the Kent-Rein complement fixation technique. Antigens tested the day following ether extraction had no preservative added. When necessary, phenyl mercuric borate to a final concentration of 1 to 50,000 was added as preservative directly after the antigen was removed from the desiccator. Antigens inactivated at 56° C. for thirty minutes on the day tested showed very little anticomplementary activity.

*Complement Fixation Technique.*—The sensitivity of the complement fixation reaction is dependent not only upon the purity of the reagents used in the test and their freedom from substances which affect the nonspecific reactivity of complement, but also on the precision with which the reagents of the hemolytic system are standardized. Spectrophotometric methods for standardization of the reagents of the hemolytic system based upon complement titrated to the 50 per cent hemolysis end point according to the instructions of Kent, Bukantz, and Rein,[8] have facilitated and increased the precision of the test. These methods have been adapted to the study of the antigenic content of the infected yolk sac membrane suspensions.

*Spectrophotometer.*—The Coleman Jr. Model 6 spectrophotometer* was used for reading all tubes of the test and for the standardization of the reagents of the hemolytic system. Kahn tubes were selected on the basis of an optical density of "O" at wave length 580 millimicrons with distilled water; such tubes were used as cuvettes in the 12 mm. adapter. Reagents in the test were adjusted to give a total volume of 2.0 ml. in the Kahn tubes.

*Diluent.*—Physiological salt solution containing magnesium sulfate and calcium chloride and buffered to pH 7.3 with barbiturate was used as saline diluent for all reagents.[7]

*Sheep's Red Blood Cells.*—A suspension containing 2 per cent by volume of washed, packed sheep cells was prepared from blood preserved by aseptic collection into an equal volume of Alsever's solution.[9] It was standardized in the spectrophotometer as described by Bukantz, Rein, and Kent.[10]

*Complement.*—Commercial† lyophilized pooled guinea pig serum was used in all the tests, the dried equivalent of 7 ml. being rehydrated by the addition of 7 ml. of the boric acid-sodium acetate diluent provided by the manufacturer rather than 5 ml. as recommended for use in the Kolmer technique. Saline containing $Mg^{++}$ and $Ca^{++}$ enhances the activity of complement so that it is necessary to start with a less concentrated guinea pig serum to obtain partial hemolysis in the range of 20 to 80 per cent when complement is titrated according to the method of Kent-Rein. The stock 1 to 20 guinea pig serum was diluted to 1 to 100 and used in 0.2 ml. to 0.45 ml. amounts in the complement titration. The unit of 50 per cent hemolysis complement was determined from a series of curves based upon dilution factors calculated from the von Krogh formula with a 1/n value of 0.2 as recommended by Kabat and Mayer.[11] This series of curves is presented in Fig. 1.

Other methods of estimating the fifty per cent hemolysis unit of complement have been described by Wadsworth, Maltaner, and Maltaner,[17] Kabat and Mayer,[11] and Stein and Ngu.[18] The use of a set of standard curves such as those presented in Fig. 1 alleviates the necessity for making graphs either arithmetric or logarithmic for each complement titration.

*Manufactured by the Coleman Electric Company, Maywood, Ill.
†Sharp & Dohme, Inc., Philadelphia, Pa.

*Hemolysin.*—Commercial* glycerinated antisheep hemolysin produced in rabbits was used in all these tests. Hemolysin was titrated in dilutions from 1:100 to 1:16,000 according to the short method of Kent.[12] The volume factor curve presented in Fig. 2 was used to calculate the units of complement required for 50 per cent hemolysis from the percentage of partial hemolysis obtained in the hemolysin titration with one 50 per cent unit of complement. The optimal dilution of hemolysin was defined as that beyond which further decrease in dilution of hemolysin failed to diminish appreciably the quantity of complement required for 50 per cent hemolysis; or the dilution of hemolysin at the point where the curve obtained by plotting the dilutions of hemolysin used in the titration as abscissa against the units of complement required for 50 per cent hemolysis as ordinate starts to form a straight line.

*Technique of the Antigenicity Test.*—The 10 per cent ether extracted infected yolk sac membrane suspensions were titrated for rickettsial antigenicity against antirickettsial rabbit

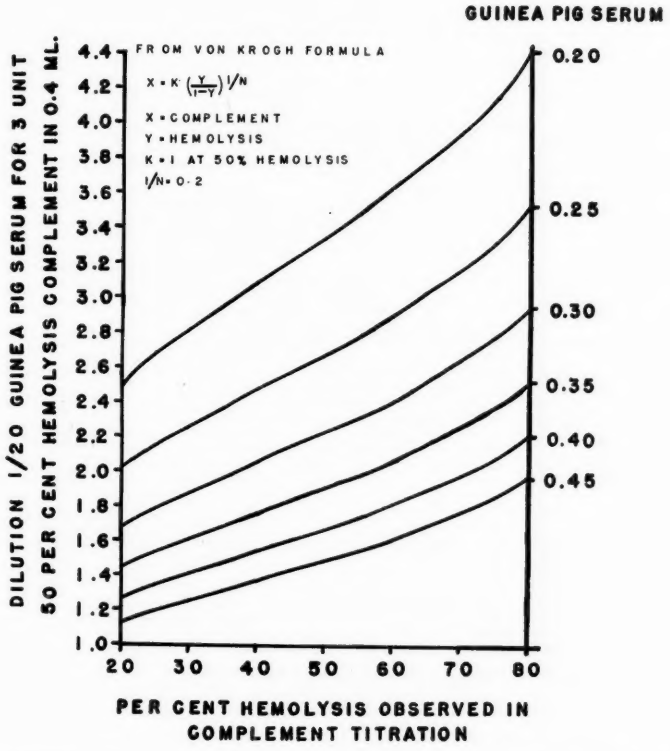


Fig. 1.—Standard curves of hemolysis for the preparation of three units of 50 per cent hemolysis complement.

The abscissa is the percentage of hemolysis obtained in the complement titration. The ordinate on the left indicates the calculated value for the denominator of the dilution of the stock 1/20 guinea pig serum needed to obtain three units of 50 per cent hemolysis complement. The quantities of guinea pig serum shown on the right side of the graph state the amount of 1/100 guinea pig serum present in each of the six tubes in the complement titration. Each curve in the graph represents degrees of hemolysis in the range from 20 to 80 per cent as calculated from the von Krogh formula using the amount of 1/100 complement present in one tube of the titration as the value of x in the formula.

*Example:* If the tube containing 0.2 ml of 1/100 guinea pig serum (uppermost curve in the graph) gave 22 per cent hemolysis, then the corresponding dilution of the stock 1/20 guinea pig serum required for 3 units of 50 per cent hemolysis complement would be 1:2.59.

Each curve is calculated from the von Krogh formula, $x = K\left(\frac{y}{1-y}\right)1/n$, where x = complement, y = per cent hemolysis, K = 1 at 50 per cent hemolysis, and 1/n = 0.2. The value of 0.2 for 1/n was obtained from the mean of the slope of regression determined in approximately 50 complement titrations; this value agrees with that found by several other workers.[8,11]

*Sharp & Dohme, Inc., Philadelphia, Pa.

serum with three 50 per cent hemolysis units of complement in 0.4 ml. amounts. The antigens were inactivated at 56° C.[13] and the antisera at 61° C.[14] for thirty minutes on the day tested. Twofold serial dilutions of the antigens from $10^{-1.0}$ to $10^{-4.0}$ were made in the barbiturate buffered saline. Duplicate tests on the antigen dilutions with antiserum and active complement were done to test the reproducibility of the results. A reagent color blank was necessary for each dilution of the antigen because the 10 per cent aqueous layer of the ether-extracted yolk sac material had a pink color which interfered with the optical density readings in the spectrophotometer. The color reagent blanks were made by adding inactive three unit com-

TABLE I. PROTOCOL OF A TYPICAL TEST

**Complement Titration**

| | Complement 1:100 in ml. | | | | | | 1:20 in ml. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.20 | 0.25 | 0.30 | 0.35 | 0.40 | 0.45 | 0.40 | 0.40 | 0.40 | 0 |
| OD | 0.070 | 0.109 | 0.210 | 0.290 | 0.350 | 0.408 | 0.490 | 0.490 | 0.490 | 0.005 |
| % | 14 | 22 | 43 | 59 | 71 | 83 | Mean = 0.490 | | | |
| OD | 0.060 | 0.108 | 0.206 | 0.270 | 0.370 | 0.408 | | | | |
| % | 12 | 22 | 42 | 55 | 76 | 83 | | | | |

**Titration Calculations***

1:20 Complement required for 3 "50 per cent units" of complement

2.04 2.08 1.98 1.98
2.04 2.07 1.94 2.04 mean = 2.02 (76 ml. 1:20 + 77 ml. saline)

**Check Titration**

| Units of Complement | 1 | 1 | 1 | 3 | 3 | 3 |
|---|---|---|---|---|---|---|
| OD | 0.270 | 0.238 | 0.250 | 0.254 | 0.240 | 0.240 |
| % | 55 | 49 | 51 | 52 | 49 | 49 |

**Titration of Antigen #232-1 D 11/4**

| Complement | | | Antigen** dilution | 1.0 | 1.3 | 1.6 | 1.9 | 2.2 | 2.5 | 2.8 | 3.1 | 3.4 | 3.7 | 4.0 | Saline |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 unit | Serum 1:100 | Positive | A OD' | 0 | 0 | 0 | 0 | 0 | 0.260 | 0.455 | 0.480 | 0.484 | 0.480 | 0.465 | 0.465 |
| | | | %" | | | | | | 53 | 92 | 97 | 98 | 97 | 94 | 94 |
| | | | B OD' | 0 | 0 | 0 | 0 | 0 | 0.152 | 0.430 | 0.464 | 0.485 | 0.485 | 0.475 | 0.500 |
| | | | % | | | | | | 31 | 87 | 94 | 98 | 98 | 96 | 100 |
| | | Neg. | C OD | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | D OD | 0.46 | 0.47 | 0.475 | 0.48 | 0.47 | 0.482 | 0.490 | 0.490 | 0.485 | 0.500 | 0.470 | 0.490 |
| | | | % | 93 | 95 | 96 | 97 | 85 | 97 | 99 | 99 | 98 | 100 | 95 | 99 |
| 1 unit | Saline | 0 \| 0.4 | E OD | 0.190 | 0.198 | 0.194 | 0.208 | 0.218 | 0.200 | 0.230 | 0.225 | 0.230 | 0.222 | 0.220 | 0.250 |
| | | | % | 38 | 40 | 39 | 42 | 44 | 41 | 47 | 46 | 47 | 45 | 45 | 51 |
| | | | F OD | 0.445 | 0.450 | 0.455 | 0.470 | 0.475 | 0.450 | 0.470 | 0.490 | 0.485 | 0.470 | 0.470 | 0.490 |
| | | | % | 90 | 91 | 92 | 95 | 96 | 91 | 95 | 99 | 98 | 95 | 95 | 99 |

Complement 1:20 OD 0.490 0.490 0.500

Standard of reference = mean = 0.493

Reagents: Date: December 12, 1949.

Hemolysin, Sharp and Dohme #81520, titrated 8-6-49, 1:2000.
Complement, Sharp & Dohme #75218-1, rehydrated to 7 ml., 1:20.
2 per cent sheep cells, collected in Alsever's 11-8-49, standardized in Coleman, Jr. spectrophotometer to OD 0.500 at 580.
Barbiturate buffered saline containing Mg++ and Ca++.
Normal rabbit serum, #49-212, inactivated at 61° C. for thirty minutes, 1:100.
Antityphus rabbit serum, #49-173, inactivated at 61° C. for thirty minutes, 1:100.
Yolk sac membrane suspension #232-1, D 11/4, epidemic typhus, Breinl strain.

*From Fig. 1.
**Log dilution denominator.
'Optical Density at 580 in Coleman, Jr. spectrophotometer.

$$\text{"Percentage of Hemolysis} = \frac{\text{OD}}{\text{Standard of references}} \times 100.$$

A,B,D, = 0.4 ml. active 3 unit complement.
C, = 0.4 ml. inactive 3 unit complement.
E, = 0.4 ml. active 1 unit complement.
A,B,C, = Serum #49-173 1:100, 0.4 ml.
D, = Serum #49-212 1:100, 0.4 ml.
F, = 0.8 ml. active 1 unit complement (2 unit).

plement to one row of tubes which contained antiserum and antigen dilution in the same amounts as were present with active three unit complement. These tubes were used to adjust the galvanometer to "O" optical density at wave length 580 millimicrons in the spectrophotometer before reading the optical density of the tubes containing the active complement.

The antigenicity test consisted of six rows of twelve tubes each, eleven tubes for the antigen dilutions, and one tube for the buffered saline diluent. Rows A, B, and C contained 0.4 ml. of 1 to 100 antirickettsial serum; this dilution provided a slight antibody excess. Row D contained 0.4 ml. of 1:100 normal rabbit serum. Row E contained 0.4 ml. of 1 unit complement and 0.4 ml. of the buffered saline diluent, and Row F, 0.8 ml. of the 1 unit complement (two units). The numbered tubes received 0.4 ml. of the same dilution of antigen, 3 ml. of each antigen dilution being required for the six tubes with a little antigen dilution left over to facilitate accurate pipetting. In addition, three tubes containing an excess of complement (0.4 ml. of 1 to 20 complement) and 0.8 ml. of the buffered saline diluent, were set up for the 100 per cent hemolysis standard of reference for use in the calculation of per-

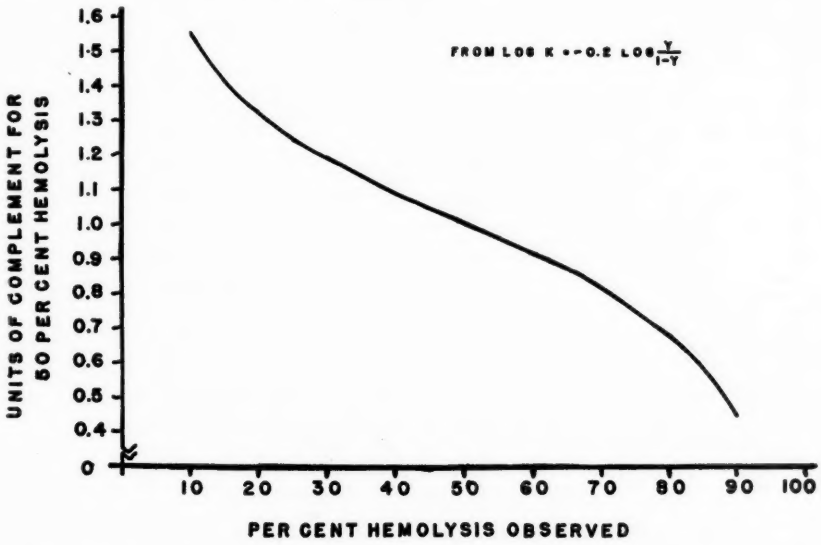


Fig. 2.—Volume factor curve for the estimation of the units of complement required for 50 per cent hemolysis from the percentage of hemolysis obtained with one unit of 50 per cent hemolysis complement. Curve calculated from the logarithmic form of the von Krogh formula, $\log x = -0.2 \log \frac{y}{1-y}$.

centage of hemolysis. Eighteen hours at 3° to 6° C. were allowed for the period of fixation of the complement, after which 0.8 ml. of 2 per cent sheep cells sensitized with the optimal dilution of hemolysin in equal amounts were added to all tubes of the test; thirty minutes at 37° C. were allowed for the period of hemolysis. All the tubes were centrifuged for ten minutes at 2,000 r.p.m. in an International Centrifuge, and the optical density of the supernatant fluid in each tube read in the spectrophotometer at wave length 580 millimicrons with the tubes of Row C used to set the galvanometer at "O" optical density.

Using the readings of the tubes with an excess of complement as the standard of reference, the percentage of hemolysis in each tube of the test was calculated by dividing its optical density by the standard of reference and multiplying the quotient by 100. These percentages of hemolysis were plotted as ordinates on cross-section paper against the corresponding dilutions of yolk sac antigen, expressed in terms of original yolk sac membrane suspension, as abscissae, and the coordinate points connected. The point at which the dilution

of yolk sac antigen corresponded with 50 per cent hemolysis was taken as the end point of the antigenicity test. This end point may be considered to be an index of the total quantity of rickettsial antigen present in the yolk sac membrane suspension.

## RESULTS

Table I contains the data from the protocol of a typical test, and Fig. 3 the graph of the results. The infected yolk sac membrane suspension in this test gave 50 per cent hemolysis end points at dilutions of $10^{-2.48}$ and $10^{-2.56}$, with the immune serum, but no reaction with the normal serum. The results of such tests are expressed as the $\log_{10}$ of the 50 per cent hemolytic end point dilution of the antigens, which is subsequently referred to as "$H_{50}A$." The values

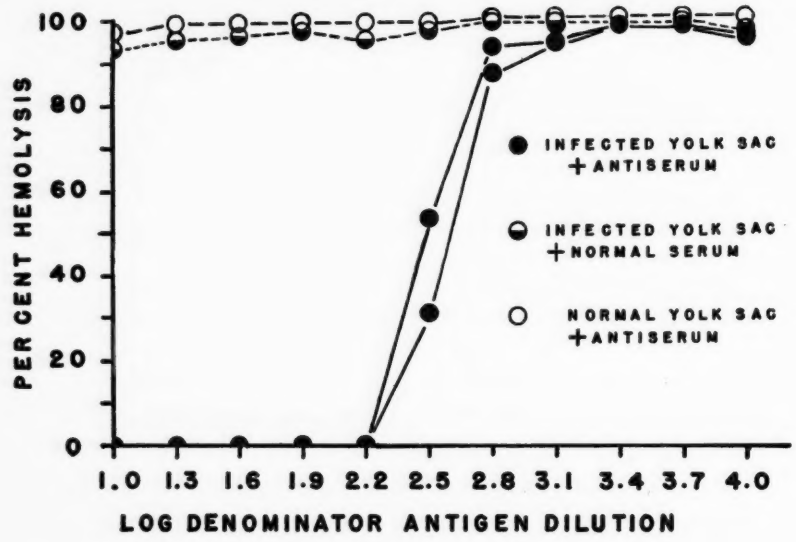


Fig. 3.—Test for Rickettsial antigen content of yolk sac suspensions.

This curve is an example of a typical test for the determination of the total content of rickettsial antigen in various yolk sac suspensions. The abscissa shows the dilutions of yolk sac suspensions as tested. The ordinate gives the percentage of hemolysis. The curves represent:

(a) typhus infected yolk sac antigen vs antityphus serum.
(b) typhus infected yolk sac antigen vs normal serum.
(c) normal yolk sac antigen vs antityphus serum.

The points for (b) and (c) show the mean values of duplicate determinations. The antigen content (50 per cent endpoint) of this particular typhus suspension is the mean value, $10^{-2.52}$, of the duplicate determinations, $10^{-2.48}$ and $10^{-2.56}$, for the typhus infected yolk sac antigen vs. antityphus serum.

from the titration in the experiment of Table I and Fig. 3 are thus stated as: $H_{50}A = 2.48$ and 2.56. A normal yolk sac membrane suspension gave no reaction with the immune serum. The antigen dilutions with one- and two-unit complement and no immune serum showed no significant anticomplementary activity. The variations in partial hemolysis obtained with the one- and two-units complement probably were due to small errors in pipetting the reagents, magnified by the sensitivity of the spectrophotometric readings which would not have been noticed in tests read by visual inspection. In the early experiments when the antigens were not inactivated before being tested, and when formalin

was added as preservative, there was some reaction between the antigen and complement which decreased with dilution but which seemed to have no effect on the $H_{50}A$ values. The substances in the antigens which give nonspecific reactions with complement seemed to be common to all the yolk sac membrane suspensions, and to be independent of the rickettsial antigen. Results obtained with the yolk sac suspensions from the same lot of embryonated eggs were within the limits of variation of the same sample.

*Effect of Viability of Rickettsiae on the Antigenicity Test.*—It is well known that rickettsiae rapidly lose viability under adverse circumstances. In order to determine whether such loss would affect the end point of the antigenicity test, a common pool of infected yolk sac membranes was divided into ten parts in sucrose PG.[3] The odd numbered specimens were placed in test tubes, the even numbered in shallow layers in Erlenmeyer flasks. Specimens 1 to 8 were placed in a water bath at 30° C. for approximately eighteen hours; during this period the oxygen tension to which the specimens were exposed was adjusted to certain levels between zero and 160 mm. Hg (room air). Specimens 9 and 10 were exposed to room air and kept at 0 to 4° C. When the eighteen-hour exposure period had elapsed, each specimen was divided into two portions; one was submitted in duplicate to the antigenicity test, the other was titrated in white mice by intravenous inoculation. The results are shown in Table II.

TABLE II. REPRODUCIBILITY OF TEST FOR ANTIGEN CONTENT OF SUSPENSIONS OF TYPHUS RICKETTSIAE (BREINL STRAIN)

| | ANTIGENICITY TEST† H50A VALUES | | | | MOUSE TOXICITY TEST‡ LD50 END POINT | | | |
|---|---|---|---|---|---|---|---|---|
| SPECIMEN* | I§ | II§ | MEAN ‖ | DIFFERENCE ‖ FROM MEAN | I§ | II§ | MEAN | DIFFERENCE¶ |
| 1 | 4.75 | 4.45 | 4.60 | 0.15 | 1.33 | 1.25 | 1.29 | 0.04 |
| 2 | 4.45 | 4.65 | 4.55 | 0.10 | 2.25 | 2.25 | 2.25 | 0 |
| 3 | 4.70 | 4.90 | 4.80 | 0.10 | 0.25 | 0 | 0.125 | 0.125 |
| 4 | 4.50 | 4.70 | 4.60 | 0.10 | 2.00 | 1.75 | 1.875 | 0.125 |
| 5 | 4.45 | 4.55 | 4.50 | 0.05 | 0 | 0 | 0 | 0 |
| 6 | 4.75 | 4.85 | 4.80 | 0.05 | 1.75 | 1.75 | 1.75 | 0 |
| 7 | 4.25 | 4.45 | 4.35 | 0.10 | 0 | 0 | 0 | 0 |
| 8 | 4.60 | 4.70 | 4.65 | 0.05 | 1.50 | 1.665 | 1.5825 | 0.082 |
| 9 | 4.65 | 4.75 | 4.70 | 0.05 | 2.25 | 2.25 | 2.25 | 0 |
| 10 | 4.65 | 4.80 | 4.725 | 0.075 | 2.33 | 2.33 | 2.33 | 0 |

*See text for description of the specimens.

†Figures refer to original yolk sac concentration expressed as $H_{50}A$ values (defined in text).

‡Figures refer to original yolk sac concentration expressed as $\log_{10}$ of denominator of the 50 per cent end point dilution, calculated by the method of Reed and Muench.[16] The mice were inoculated by Dr. J. C. Snyder.

§Columns headed I and II show values of duplicate determinations.

‖The mean $H_{50}A$ value for the antigenicity tests is 4.628 with a standard deviation of 0.197 between specimens, and 0.125 between replicates.

¶The standard deviation of the difference between replicates for the mouse toxicity tests is 0.089.

The toxicity of the typhus rickettsiae was greatly affected by the exposure to low oxygen tension as indicated in the tests with mice by the change in $LD_{50}$ values of more than two hundred fold in specimens 5 and 7. However, this change did not affect the results of the tests for total rickettsial antigen, as

shown by the figures in the first two columns of Table II. The mean $H_{50}A$ was 4.628 and the standard deviation, 0.197 between specimens, 0.125 between replicates.

*Reproducibility of the Antigenicity Tests.*—The data in Table II indicate an acceptable degree of reproducibility of duplicate determinations in that particular experiment. In order to determine the reliability of the method over a period of several months, titrations were made of various epidemic and murine antigen preparations. There were eighty-seven batches which were tested. The mean difference of the eighty-seven duplicate determinations of $H_{50}A$ values from their means was 0.019, standard deviation, 0.037; this degree of variation is quite acceptable for this type of test.

*Sensitivity of the Test.*—Infected yolk sac membrane antigens were diluted with different saline diluents or with normal yolk sac membrane suspensions to provide a series of specimens with known differences in antigen content. These mixtures were subjected in duplicate to titration by the antigenicity test as a measure of its sensitivity. Data from these experiments are presented in Table III. The observed $H_{50}A$ values corresponded very closely to the quantity of

TABLE III. SENSITIVITY OF THE ANTIGENICITY TEST

| POOL NO.* | SAMPLE | DILUTION | DILUENT | $H_{50}A$† | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | OBSERVED | | | THEORETICAL‡ | DIFFERENCE: THEORETICAL MINUS MEAN OF OBSERVED VALUES** |
| | | | | I | II | MEAN | | |
| 2829 | 1 | 1:10 | § | 3.12 | 3.15 | 3.135 | 3.11 | −0.025 |
| | 2 | 1:10 | § | 3.11 | 3.05 | 3.08 | 3.11 | +0.03 |
| | 3 | 1:10 | ‖ | 3.10 | 3.13 | 3.115 | 3.11 | −0.005 |
| | 4 | 1:10 | ‖ | 3.10 | 3.10 | 3.10 | 3.11 | +0.01 |
| 3844 | 5 | 1:10 | ¶ | 3.20 | 3.10 | 3.15 | 3.15 | −0.0 |
| | 6 | 1:20 | ¶ | 2.95 | 2.95 | 2.95 | 2.85 | −0.10 |
| | 7 | 1:40 | ¶ | 2.65 | 2.75 | 2.70 | 2.55 | −0.15 |
| | 8 | 1:80 | ¶ | 2.20 | 2.20 | 2.20 | 2.25 | +0.05 |
| 3844 | 9 | 1:20 | § | 2.94 | 2.94 | 2.94 | 2.85 | −0.09 |
| | 10 | 1:80 | § | 2.20 | 2.20 | 2.20 | 2.25 | +0.05 |
| | 11 | 1:200 | § | 1.75 | 1.75 | 1.75 | 1.85 | +0.10 |
| | 12 | 1:800 | § | 1.75 | 1.20 | 1.325 | 1.25 | −0.075 |

*Pool No. refers to the particular lot of typhus infected yolk sac membrane from which dilutions were made for the various samples.

†"$H_{50}A$" is defined in the text.

‡The "theoretical" values were derived as follows: the $H_{50}A$ values of the pool were determined in several independent tests; this mean was then used to calculate the expected value for the particular dilution of each sample.

§Physiological saline buffered to pH 7.3 with M/100 phosphate.

‖Barbiturate buffered saline.[7]

¶Ten per cent normal yolk sac suspension in barbiturate buffered saline.[7]

**The mean of the figures in this column is 0.017, standard deviation 0.074.

infected yolk sac in the specimens as indicated by the last column in the table. The mean difference between observed and theoretical values was 0.017, standard deviation, 0.074.

*Comparison of the 50 per cent Hemolytic End Point Method and the Conventional Antigen Titration.*—To determine the difference in sensitivity between the technique as herein described and the conventional complement fixation

TABLE IV. COMPARISON OF THE CONVENTIONAL COMPLEMENT FIXATION TEST AND THE 50 PER CENT HEMOLYSIS TEST

| TYPHUS INFECTED YOLK SAC ANTIGEN | TECHNIQUE* CONVENTIONAL† | 50% HEMOLYSIS | DIFFERENCE‡ |
|---|---|---|---|
| 990–3 | – | 1.160 | 1.160 |
| –6 | – | 1.450 | 1.450 |
| –10 | – | – | – |
| –12 | – | – | – |
| 954–2 | 1.9 | 2.700 | 0.800 |
| –8 | 1.6 | 2.250 | 0.650 |
| –12 | 1.3 | 2.100 | 0.800 |
| –14 | 1.9 | 2.600 | 0.700 |
| –15 | 1.6 | 2.200 | 0.600 |
| –16 | 1.6 | 2.150 | 0.550 |
| 2873–1 | 1.6 | 2.350 | 0.750 |
| –5 | 1.6 | 2.350 | 0.750 |
| –6 | 1.0 | 2.275 | 1.275 |
| –7 | 1.3 | 2.050 | 0.750 |
| –8 | 1.3 | 1.925 | 0.625 |
| 2874–1 | 1.9 | 2.980 | 1.080 |
| –5 | 1.3 | 2.570 | 1.270 |
| –6 | 2.2 | 3.050 | 0.850 |
| –7 | 1.9 | 2.700 | 0.800 |
| –8 | 1.9 | 3.000 | 1.100 |
| 2875–1 | 1.6 | 2.530 | 0.930 |
| 2878–1 | – | – | – |
| –2 | – | 1.250 | 1.250 |
| –3 | 1.3 | 1.925 | 0.625 |
| –4 | 1.3 | 2.215 | 0.915 |
| –5 | 1.6 | 2.100 | 0.500 |
| 2879–1 | – | – | – |
| –2 | – | – | – |
| –4 | 1.6 | 2.275 | 0.675 |
| 2881–2 | – | 1.150 | 1.150 |
| –3 | – | 1.100 | 1.100 |
| –5 | – | 1.450 | 1.450 |
| 2885–1 | – | 1.725 | 1.725 |
| –4 | – | – | – |
| –5 | 1.3 | 1.925 | 0.625 |

*$\text{Log}_{10}$ of the denominator of the antigen dilution.
†The conventional test referred to is described in Reference 16.
‡Mean and standard deviation of figures in this column: 0.928, ± 0.308.

test,[16] thirty-five batches of typhus-infected antigen were titrated by both techniques. In every instance the two types of test were performed on a given antigen on the same day. The results are summarized in Table IV.

There were six samples of the thirty-five which contained too small a quantity of antigen for detection by either test, and six which were detected only by the 50 per cent hemolysis test.

In comparing the two tests, it should be recalled that values for the end point of the antigenicity test in all this work was taken as the dilution of yolk sac suspension before it was added to the other reagents of the test. After all reagents were added the yolk sac antigen was further diluted 1:5 in the 50 per cent hemolysis test as herein described and 1:6 in the conventional technique. Thus a correction factor of 0.079 should be applied to the mean of the differences shown in the last column of Table IV. This brings the figure to 0.849, which indicates that the 50 per cent hemolysis test affords a mean increase of seven fold in sensitivity for the detection of typhus antigen.

## DISCUSSION

The complement fixation technique as modified in this paper depends upon the use of specific antisera free from nonspecific antibodies. The test is facilitated by the use of reagents standardized in a spectrophotometer to a 50 per cent hemolysis unit of complement with the aid of standard curves of hemolysis. Subjective factors in reading the reactions of the tests have been eliminated by adjusting the reagents to a total volume of 2.0 ml. so that all the tests can be read spectrophotometrically.

This test for antigenicity was developed for the study of factors affecting the growth of pathogenic rickettsiae in embryonated hens' eggs, where a reliable, rapid, quantitative technique is required. The important feature of this technique is that the amount of ether-extracted rickettsial antigen present in the infected yolk sac membrane is correlated closely with the total numbers of rickettsiae present; the antigenicity of a suspension as determined by this technique is a measure of the numbers of rickettsiae present at a given time, and is not affected by the death of rickettsiae between the time of harvesting and the time of testing the infected material as are the usual tests which depend upon the presence of viable rickettsiae for their end points. It must be emphasized, however, that although this technique increases the sensitivity by fivefold to tenfold over the conventional technique for titration of antigens, it is necessary to have present a relatively high concentration of rickettsial antigen for evaluation by this technique.

## SUMMARY

A complement fixation technique which employs a spectrophotometer for the standardization of the reagents of the hemolytic system and uses complement units based upon 50 per cent hemolysis has been adapted to quantitative studies of the antigenicity of yolk sac membranes infected with typhus rickettsiae. Duplicate determinations on eighty-seven samples of yolk sac membrane suspensions showed a highly satisfactory degree of reproducibility. The test permits an increase in sensitivity of seven fold over the conventional tests based upon complement titrated to 100 per cent hemolysis.

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# A MICRODIFFUSION METHOD FOR THE DETERMINATION OF NITROGEN LIBERATED AS AMMONIA

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CONWAY and Byrne[1] have devised a simple diffusion cell to measure ammonia in amounts of 10 or more micrograms per aliquot. They have described its versatility and its use in conjunction with ammonia-liberating reactions to determine ammonia, urea, and total nitrogen.[2-6] However, it has some disadvantages: (a) in many cases, a reaction vessel is needed in addition to the diffusion cell; (b) the cell is comparatively expensive; (c) removal of the heavy greases used to make the cell airtight provides a cleaning problem in that strong alkalies and acids used in cleaning may leave traces which can vitiate the results of the microtitration involved; (d) in adapting the method to colorimetry an additional transfer is required.

Among the early modifications of Conway's method was one by Borsook[7] which determined as little as 0.5 $\mu$g of ammonia nitrogen. Needham and Boell,[8] and Thompkins and Kirk[9] have each devised a special apparatus to digest and diffuse minute amounts of total nitrogen (0.5 to 20 $\mu$g). Hawes and Skavinski[10] described a basically similar procedure, simplified by using a test tube for the digestion and diffusion vessel. The range was 10 to 100 $\mu$g of nitrogen. Conway[11] has critically reviewed these procedures. Sobel and his associates[12] have used a modified Cullen-Van Slyke aerator to measure 5 to 100 $\mu$g of ammonia nitrogen liberated by various reactions, and the method has since been modified.[13] The ammonia liberated was measured by a micro-titration procedure. Shaw and Beadle[14] reported excellent precision in another modification involving titration after diffusion into the acid on an inverted glass slide.

In the method to be described ammonia is separated by diffusion and then measured colorimetrically. Having the ammonia-liberating reactions take place within the diffusion vessel reduces manipulation and contamination.

## METHODS

*Determination of Ammonia.—*

Reagents:

(a) Saturated potassium carbonate solution
(b) Standard ammonia solution containing 100 $\mu$g N per milliliter (0.4716 Gm. $[NH_4]_2SO_4$ per liter)
(c) 1 N $H_2SO_4$ in dropping bottle
(d) Nessler's Solution diluted 1:10 before use

Apparatus:

(a) Penicillin bottles with footed glass rod (Fig. 1)
(b) 0.2 ml. pipette (Hagedorn-Jensen blood sugar type)
(c) Rotator (Fig. 2)

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If the saturated potassium carbonate is stored in a flask capped with a rubber stopper containing a footed glass rod moistened with one drop of 1 N $H_2SO_4$, its ammonia blank will be negligible. Commercial Nessler's reagent (Paragon) has been used. The penicillin bottles were those discarded by hospital wards. The rubber stopper belonging to the penicillin bottle was bored to fit a footed glass rod (Fig. 1).

*Procedure:* 0.2 ml. of standard ammonia solution is pipetted into a penicillin bottle, allowing thirty seconds for drainage, and then blowing the remaining contents of the pipette on the side of the bottle. The pipette is used for all samples and is rinsed with each new sample before use. One milliliter of saturated potassium carbonate is added, and a drop (about 0.03 ml.) of 1 N $H_2SO_4$ is placed on the footed glass rod, and the stopper and rod are replaced in the bottle. The bottle is put on the rotator, and the liberated ammonia is allowed to diffuse for the desired length of time. The rod, which now carries ammonium sulfate, is removed, then washed off with 10.0 ml. of diluted Nessler's solution, from a pipette into the desired container. After standing five minutes at room temperature, the intensity of color is measured in a suitable photoelectric colorimeter using a 420 filter. The blank was prepared by replacing the sample with distilled water and Nesslerizing the diffusate.

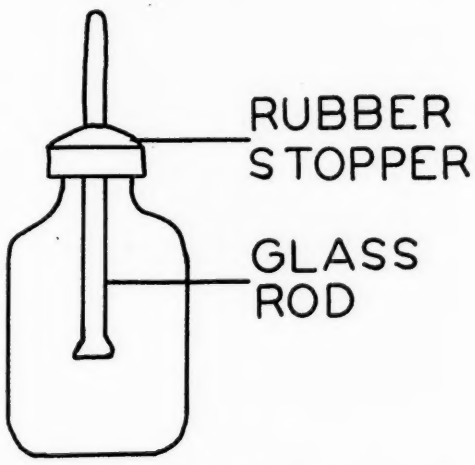


Fig. 1.—Diffusion cell made from a penicillin bottle (6 cm. in height; outside diameter at base, 3 cm.; contains approximately 25 milliliters).

TABLE I. MEASUREMENT OF AMMONIA IN URINE AND RECOVERY OF ADDED AMMONIA

| SAMPLE | $NH_3$-N FOUND | SAMPLE | $NH_3$-N FOUND |
|---|---|---|---|
| | μg | | μg |
| 0.2 ml. of urine diluted 1:5 | 21.6 | 0.2 ml. of same urine + 6.6 μg $NH_3$-N | 28.3 |
| | 22.1 | | 28.6 |
| | 22.2 | | 28.8 |
| Average | 22.0 | | 28.6 |

Recovery—6.6 μg; 100 per cent.

*Nesslerization:* Fig. 3, curve *A*, demonstrates the relationship between color intensity and quantity of ammonia, ranging from 5 to 30 μg per 10 ml. of final solution. In curve *B*, the same quantities of ammonia were isolated by diffusion. The curve deviates slightly from a straight line as has been reported,[15] but at most this is less than 2 per cent from an assumed straight line drawn through the points 0 and 20 micrograms. For the usual analytical work the standard curve may be established by the line connecting the optical density of the blank and 20 μg of ammonia. More precise data are acquired by using

standard solutions which approximate the concentration of the unknown samples. Another procedure for providing a standard curve which has proved useful and accurate is to establish knowns at 10 and 20 μg levels. The readings have been constant enough to permit the use of a single standard curve without daily repetition of standards when errors within 5 per cent are permissible. The temperature of Nesslerization and the time of the reaction affect the color intensity achieved. These factors should be kept in mind when daily variations are encountered.[16]

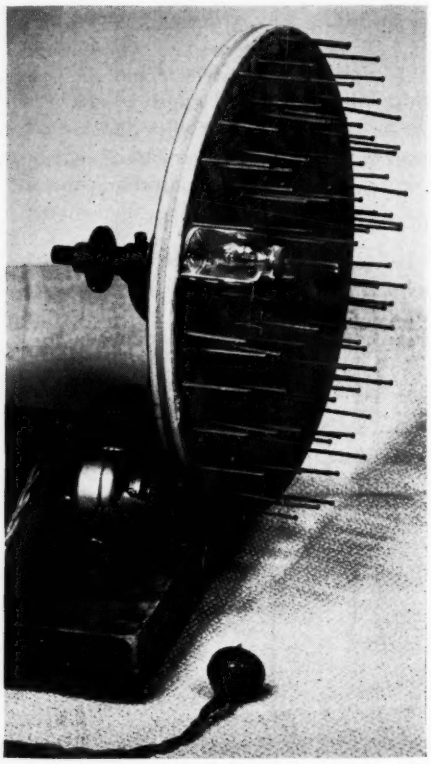

Fig. 2.—Apparatus for rotating the bottles to accelerate diffusion of ammonia.

*Diffusion:* Maximum diffusion on standing is obtained in eighteen to twenty-four hours. If the cells are rotated in the machine (Fig. 2), maximum diffusion occurs in thirty minutes. Fig. 3, curve *B*, demonstrates a color concentration curve obtained by one hour of rotation. This curve almost exactly coincides with an eighteen-hour diffusion curve without rotation (not charted).

Overnight diffusion is disadvantageous, (a) when results are needed quickly as in clinical work, and (b) when the contents of biological fluids in alkaline solution may undergo hydrolysis and yield additional ammonia. A rocking device with about 150 pulses per minute gives satisfactory results, but is slower than the rotator. Fig. 4 shows the time-diffusion curves for bottles rotated at 19 and 54 r.p.m., both speeds giving maximum diffusion of ammonia in forty-eight minutes or less. If urgency is great, knowns and unknowns can be rotated for as little as twelve minutes and measurements made at this time. For routine work diffusion at 40 r.p.m. for thirty minutes has proved suitable although other combinations may be equally satisfactory.

*Quantity of Ammonia Nitrogen Measured.*—Using the Evelyn colorimeter with the 420 filter, amounts of 5 to 25 μg (in 10 ml. Nessler's solution) give readings of 71 to 24

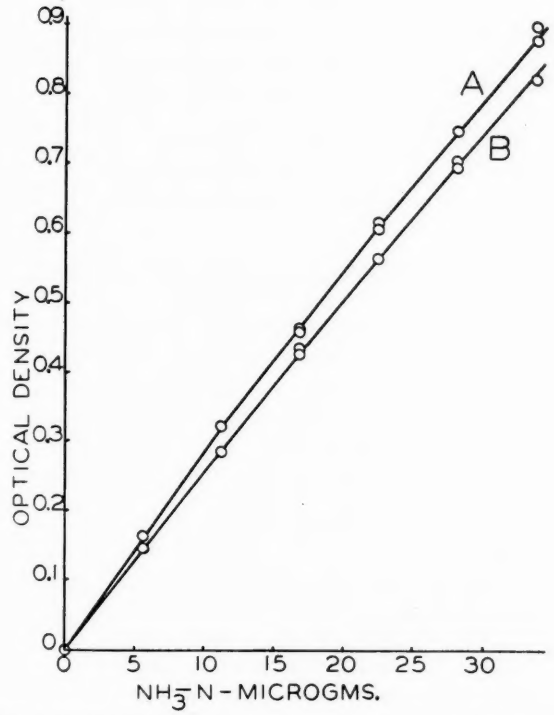


Fig. 3.—Color-concentration curves of standard ammonia solutions Nesslerized before (*A*) and after (*B*) diffusion in the rotator for one hour at room temperature. Measurements were made in the Evelyn photoelectric colorimeter using the 420 filter and 10 ml. of Nesslerized solution.

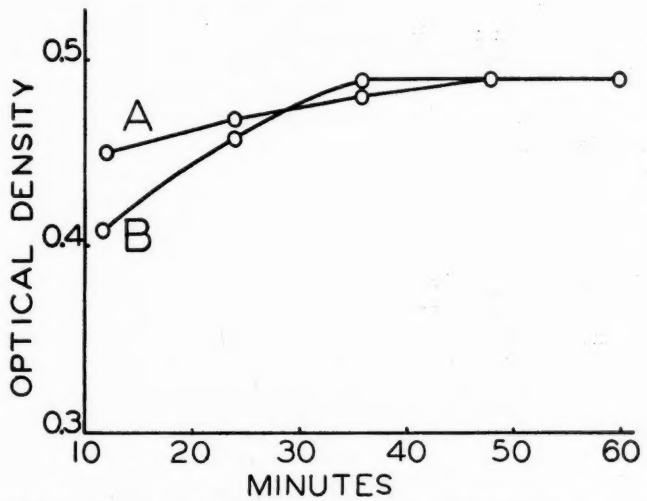


Fig. 4.—The influence of speed of rotation on the recovery of ammonia by diffusion. The penicillin bottles each contained 19.7 μg of $NH_3$-N, and were rotated at 19 r.p.m. (Curve *A*) and 54 r.p.m. (Curve *B*).

per cent transmittance. The range of the method can easily be extended from 2 to 200 μg or more of nitrogen by dilution of the sample or of the diffusate.

*Measurement of Ammonia in Urine or Other Biological Fluids.*—0.2 ml. of diluted urine or biological fluid containing 10 to 25 μg of ammonia nitrogen is placed in a penicillin bottle. 1.0 ml. of saturated potassium carbonate is added and the bottles are rotated for thirty minutes. The isolated ammonia is measured by the addition of Nessler's solution as described. Standard solutions receive similar treatment. Table I gives the results of an analysis of urine and shows the recovery of added ammonia.

*Measurements of Total Nitrogen by Adaptation of Kjeldahl Digestion.*—

Reagents:

(a) Digestion mixture: $H_2SO_4$, 3 mol; $K_2SO_4$, 0.6 mol., and $HgSO_4$, 0.017 mol. per liter.
(b) Wash solution. Sodium Thiosulfate, 0.05 mol. and NaOH 1 mol. per liter.
(c) NaOH pellets.

The Streptomycin bottle which is similar to the penicillin bottle except that it is longer and narrower (27 by 68 mm.) has been used as the digestion flask. It is also available in hospitals as a discard. Its advantage lies in its smaller diameter which permits the use of only 0.5 ml. of digestion mixture to cover its flat base and occupies less space on the hot plate than the ordinary penicillin bottle. It can be fitted into the clamp of the rotator, which is made for the penicillin bottle, by applying a strip of felt 100 mm. long, 38 mm. wide and 2 mm. thick.

*Digestion Procedure:* Serum or urine are diluted 1:100 with ammonia-free water and 0.2 ml. of sample, containing about 10 to 25 micrograms of nitrogen, is placed in a digestion bottle. Other volumes may be used but small volumes reduce the time required for evaporation of water. Then 0.5 ml. of digestion mixture is added and the bottles placed on the periphery of an electric hot plate covered with a 1/16 inch thick asbestos pad. The digestion may be carried out in an open room; boiling is avoided. When the water is completely gone as evidenced by the disappearance of droplets from the sides of the bottle, and fumes begin to rise, the bottle is capped with a marble. The temperature is raised so that the sulfuric acid refluxes gently on the sides of the bottle. When digestion is complete the heating is discontinued and the bottle is allowed to cool slowly, thus reducing breakage to less than one bottle in a hundred determinations. The marble is lifted with tweezers and washed with the alkaline-thiosulfate solution, so that it runs into the flask. The sides of the upper part of the flask are rinsed with the same solution using a total of about 1.5 to 2.0 ml. from a small wash bottle. Three pellets of sodium hydroxide are dropped in each bottle which is then closed with a stopper holding a footed glass rod from which is suspended one drop of dilute sulfuric acid. The flasks are put on the rotator for sixty to seventy-five minutes. The ammonia, isolated by diffusion, is Nesslerized as described above and compared against standards which have been digested in the same manner as the unknowns.

The heating time for complete digestion varies according to the nature of the nitrogen compounds. Urine is adequately digested after one-half hour of refluxing, whereas serum darkens in that time and clears after one hour of refluxing. Good results have been obtained on serum with a total of two hours of digestion; i.e., forty-five minutes for evaporation and seventy-five minutes of refluxing.

The optimum period of rotation was determined by the recovery of standard ammonium sulfate solutions added to the digestion mixture after rotation for varying periods. The data are similar to the results shown in Fig. 4, with the exception that the plateau begins at sixty minutes. Table II gives the total nitrogen of samples of urine and serums measured on different days and compared against the micro-Kjeldahl procedure of Hiller and associates.[17] Measurements by the latter procedure are made on 1000 μg of nitrogen. In addition the estimated protein content of three serums measured by the method herein described is compared with independent measurements made by another observer.

*Determination of Ammonia-Liberating Compounds.*—Urea, hydrolyzed by urease, can be measured by its quantitative liberation of ammonia in the diffusion cell herein described. The procedure of Conway[11] for hydrolysis is convenient.

Amino acids under the conditions described by Schlenker[18] will react with ninhydrin and then in strongly alkaline solution will liberate stoichiometric amounts of ammonia. This procedure applied to the microdiffusion cell gives accurate measurements of some pure amino acids. The procedure fails in biological solutions because compounds other than amino acids also contribute ammonia. Glutamine has been measured by this procedure using glutaminase[19] for hydrolysis.

TABLE II. MEASUREMENT OF TOTAL NITROGEN IN URINE OR PROTEIN IN SERUM IN GRAMS PER 100 MILLILITERS

| SAMPLE | TOTAL NITROGEN | | TOTAL PROTEIN | |
|---|---|---|---|---|
| | HILLER, ET AL.[17] | THIS METHOD | HILLER, ET AL.[17] | THIS METHOD* |
| #1 Urine | 1.244 | 1.250 | | |
| | 1.243 | 1.236 | | |
| Average | 1.244 | 1.243 | | |
| #2 Serum | | | | 7.28 |
| | | | | 7.34 |
| | | | | 7.26 |
| | | | | 7.35 |
| Average | | | 7.20 | 7.22 |
| | | | 7.27 | |
| | | | 7.20 | |
| | | | 7.22 | 7.29 |
| #3 Serum | | | | 7.50 |
| | | | | 7.45 |
| | | | | 7.44 |
| | | | 7.54 | 7.48 |
| Average | | | 7.54 | 7.47 |
| #4 Serum | | | 6.08† | 5.95 |
| #5 Serum | | | 6.52† | 6.35 |
| #6 Serum | | | 6.15† | 6.18 |

*Samples of urine and serum diluted 1:100 to contain about 20 micrograms N per 0.2 ml.

†Performed independently by Dr. Reinhold, Hospital of the University of Pennsylvania. For samples #1, 2 and 3, each line represents analyses done on different days, the intervals ranging from 3 to 8 days.

## DISCUSSION

This method depends upon the liberation of ammonia, its separation by diffusion, and measurement by Nesslerization; all with simple equipment. The aliquots used may be varied to contain from a few to several hundred micrograms of ammonia-nitrogen. Its applicability is demonstrated in the measurement of the compounds described above. Three forms of nitrogen (ammonia, urea, and total nitrogen) may be measured in biological fluids. Other applications involving the liberation of ammonia stoichiometrically are possible. The procedure has been carried out by several persons, and found suitable for routine use.

## SUMMARY

A microdiffusion method for the determination of ammonia, which employs a simple diffusion cell and a mechanical rotator, is described. Total nitrogen is readily measured using the diffusion cell as a digestion vessel first

and as a diffusion chamber later. The procedure can be adapted to the measurement of urea and other compounds which liberate ammonia stoichiometrically. The range can be adjusted from 2 to 200 μg of nitrogen; the most convenient range being 10 to 25 micrograms. In our hands this method has proved simple, rapid, and accurate.

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## Erratum

In the article, "Further Studies on Recovery From Radiation Injury," by Dr. Leon Jacobson and associates, in the May issue of the Journal, on page 692, the first figure in Table VII under per cent surviving at 28 days should read "80," instead of "0."